1220644

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

September 02, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/513,922*
FILING DATE: *October 23, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/10564*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

PTO/SB/16 (08-03)
Approved for use through 7/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Expr ss Mail Label N . EV 309882836 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Roy H.L. Robert A. Dona	Pang Wiercinski Hevroni	Etna, New Hampshire Lincoln, Massachusetts Lexington, Massachusetts

Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

POROUS PARTICULATE COLLAGEN SPONGES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

[X] Customer Number: 00959

OR

[ ] Firm or Individual Name	Amy E. Mandragouras LAHIVE & COCKFIELD, LLP				
Address	28 State Street				
City	Boston	State	MA	Zip	02109
Country	US	Telephone	(617) 227-7400	Fax	(617) 742-4214

ENCLOSED APPLICATION PARTS (*check all that apply*)

[X] Specification *Number of Pages* 90 [ ] CD(s), Number

[X] Drawing(s) *Number of Sheets* 60 [ ] Other

[X] Application Data Sheet. See 37 CFR 1.76 (specify):

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check or money order is enclosed to cover the filing fees.

[X] The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 12-0080

[ ] Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____

TYPED OR PRINTED NAME Amy E. Mandragouras

REGISTRATION NO. *(if appropriate)* 36,207

TELEPHONE (617) 227-7400

Date October 23, 2003

Docket Number: GRN-005-2

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 309882836 US, in an envelope addressed to: MS Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: October 23, 2003 Signature: _____ (Amy E. Mandragouras)

Use in lieu of PTO/SB/17 (08-03)
(Form updated to reflect FY 2004 fees effective 10/1/03)

FEE TRANSMITTAL for FY 2004

Effective 10/01/2003, Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Roy H.L. Pang
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	GRN-005-2

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 12-0080

Deposit Account Name: Lahive & Cockfield, LLP

The Director is authorized to: *(check all that apply)*

☒ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s) during the pendency of this application

☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160.00

SUBTOTAL (1) ($) 160.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	=	Fee Paid
Total Claims	-** =		x		=	
Independent Claims	-** =		x		=	
Multiple Dependent					=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge – late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for ex *parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
Other fee (specify)					

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0.00

SUBMITTED BY

(Complete *(if applicable)*)

Name (Print/Type)	Amy E. Mandragouras	*Registration No. (Attorney/Agent)*	36,207	*Telephone*	(617) 227-7400
Signature				*Date*	October 23, 2003

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 309882836 US, in an envelope addressed to: MS Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: October 23, 2003 Signature: _____ (Amy E. Mandragouras)

Application No. (if known):

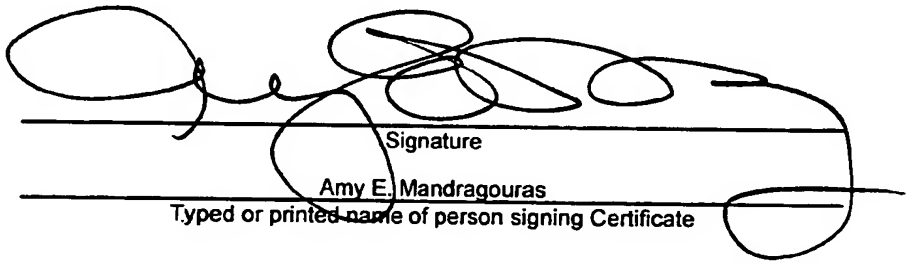
Attorney Docket No.: GRN-005-2

Certificate of Express Mailing Under 37 CFR 1.10

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail, Airbill No. EV 309882836 US in an envelope addressed to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

on October 23, 2003
Date



Signature

Amy E. Mandragouras
Typed or printed name of person signing Certificate

Note: Each paper must have its own certificate of mailing, or this certificate must identify each submitted paper.

New Provisional Patent Application consisting of 71 pages of specification, 18 pages of claims, Abstract 1 page, 60 sheets of drawings (Figures 1A-1C, 1C-2nd Batch, 2, 2A-2B, 3, 3A-3B, 4, 4A-4E, 4- 2nd Batch, 4A-4B – 2nd Batch, 5, 5A-5B, 6, 6A, 7, 9, 10, 11, 12, 13, 14, 23, 23A-23B, 24,24, 25, 26, 25, 16 24, 25, 26, 27, 28, 29, 33, 34, 35, 37A-37B, 38 41, 39, 42, 51, 50, 52, 53, 54, 54 (cont-1), 54 (cont-2) 55, 56, 57 and 58;

Provisional Patent Application Transmittal (1 page)
Application Data Sheet (2 pages)
Fee Transmittal (1 page, in duplicate)
This Certificate of Express Mailing (1 page)
Prepaid acknowledgement postcard

POROUS PARTICULATE COLLAGEN SPONGES

RELATED APPLICATIONS

This application is related to U.S. Provisional Application 60/460341, filed on April 4, 2003, entitled "Porous Particulate Collagen Sponges"; U.S. Provisional Application 60/370,043, filed on April 4, 2002, entitled "Tissue Composites and Uses Thereof"; and PCT Application PCT/US03/10439 filed on April 4, 2003, entitled "Tissue Composites and Uses Thereof," the contents of which are hereby incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Injuries to soft tissues are extremely common in hospital clinics. In fact, soft tissue replacements amount to an estimated 35% of the world market for all medical devices (Materials Technology Foresight in Biomaterials, Institute of Materials, London (1995).

There have been many options proposed for the repair of soft tissues. These generally involve synthetic materials, biological materials or a combination of the two. Synthetic alternatives have demonstrated *in vivo* instability, and thus relatively poor long-term performance. Biological solutions traditionally involve autografts, allografts or xenografts, depending on the source of tissues. Each of these options has proved to be far from ideal with, for example, autografts leading to donor site morbidity, and allografts and xenografts to graft rejection.

The relative failure of many surgical, synthetic and graft solutions has led to a growing interest in the development of cell-seeded or tissue-engineered repair systems to address a number of clinical problems related to tissues, *e.g.*, connective tissue or soft tissue. Such repair systems typically involve autologous or allogenic cells that are isolated from a tissue biopsy at a site remote to the injury. Typically, the isolated cells are expanded in cell culture and seeded in a suitable three-dimensional scaffold material, which when implanted into the injured site elicits a biological repair.

While previous studies have examined collagen sponges or foams for use as hemostatic agents, more recent attempts have examined collagen scaffolds for tissue repair *in vivo*, and as research tools *in vitro* for seeding various cell types in the study of cell function in three dimension (see *e.g.*, U.S. Patent No. 5,709,934). As collagen sponges have a low immunogenicity, and consist of a naturally occurring structural protein, cells can attach, interact with and degrade scaffolds of this type. The sponges are usually cross-linked to

provide the degree of wet strength and measured resistance to dissolution needed for therapeutic efficiency.

Collagen is a preferred material for tissue engineering because the extracellular matrix of animal tissue comprises a sponge-like collagen network. However, it has been difficult to create a man-made, sponge-like collagen network, from purified insoluble or soluble collagen obtained from an animal source that mimics the natural extracellular matrix. Man-made sponges in various forms, including sheets and particulates are known, but have not exhibited the most desirable combination of properties, *e.g.*, resorbability, no toxicity, and satisfactory porosity, particularly when wetted in an aqueous medium.

Man-made collagen sponges are typically cross-linked, otherwise, the sponge-like structure would collapse upon wetting. Typical chemical cross-linking agents, like gluteraldehyde, have been used to prepare collagen sponges. Using chemical cross-linking agents, it is possible to tailor formulations such that the sponge can be wetted directly into an aqueous medium without collapsing the porous structure; however, such agents are toxic, and sponges cross-linked with external agents may not be easily resorbable. In addition, while collagen sponges are known that have been dehydrothermally cross-linked to overcome problems with toxicity and resorbability, both the large pore size and shrinkage/reduction of porosity that occurs upon wetting directly in an aqueous medium have not reached the sought after paradigm in tissue engineering.

SUMMARY OF INVENTION

The present invention solves these problems. The objective of the present invention is the development of new porous particulate collagen sponges, combining the desirable features of low toxicity, resorbability, and satisfactory porosity, particularly when wetted in an aqueous medium. Accordingly, the present invention is directed to new porous, particulate, dehydrothermally cross-linked, wetted sponges, as well as a process for making them.

One embodiment of the present invention are spherical, porous, dehydrothermally cross-linked, collagen sponges prepared by the methods of the invention.

Another embodiment of the present invention are wetted, spherical, porous, dehyrdothermally cross-linked, collagen sponges.

Another embodiment of the present invention are particulate, non-spherical, porous, dehyrdothermally cross-linked, collagen sponges prepared by the methods of the invention.

Another embodiment of the present invention are wetted, porous, dehydrothermally cross-linked, collagen sponges of any shape or size (wherein porosity, shape, volume, morphology etc. is maintained vs dry precursors).

Another embodiment of the present invention are wetted, particulate, non-spherical, porous, dehydrothermally cross-linked, collagen sponges.

Another embodiment of the present invention are small, *e.g.*, less than 50μ, particulate, porous collagen sponges

Another embodiment of the present invention are small, *e.g.*, less than 50μ, wetted, particulate, porous, dehydrothermally cross-linked collagen sponges

Another embodiment of the present invention are processes to produce small, *e.g.*, less than 50μ, particulate, porous collagen sponges

Another embodiment of the present invention are processes to produce small, *e.g.*, less than 50μ, wetted, particulate, porous, dehydrothermally cross-linked collagen sponges

Another embodiment of the present invention is a particulate, non-spherical, porous, dehydrothermally cross-linked, collagen sponge prepared by the methods of the invention, wherein pore size and particle size can be tailored.

Another embodiment of the present invention is a process for producing particulate, non-spherical, porous, dehydrothermally cross-linked collagen sponges wherein pore size and particle size can be tailored.

Another embodiment of the present invention is a process for producing particulate, non-spherical, porous, dehydrothermally cross-linked, collagen sponges wherein particle size can be controlled over a narrow range.

Another embodiment of the present invention is a process for producing spherical, porous, dehydrothermally cross-linked collagen sponges.

Another embodiment of the present invention relates to processes for producing wetted, dehydrothermally cross-linked collagen sponges comprising a morphology that is similar to that for the dry precursors.

Another embodiment of the invention relates to equipment, wherein grinding and separation of particles, by particle size, is done simultaneously to produce fractionated, particulate, non-spherical collagen sponges.

Another embodiment of the invention relates to engineered tissue composites comprising cells and the wetted, dehydrothermally cross-linked collagen sponges of the present invention.

Another embodiment of the invention relates to engineered tissue composites comprising cells, gel, and the wetted, dehydrothermally cross-linked collagen sponges of the present invention.

Another embodiment of the invention relates to micro-carrier composites comprising

cells and the wetted, dehydrothermally cross-linked, collagen sponges of the present invention.

Another embodiment of the invention is an enclosure comprising the micro-carrier composites of the present invention

Another embodiment of the invention relates to micro-carrier composites comprising cells and the wetted, dehydrothermally cross-linked collagen sponges of the present invention wherein each sponge is encased in a complex coacervate gel

Another embodiment of the invention is a cell-based drug delivery device comprising wetted collagen particles of the present invention.

Another embodiment of the invention is a cell-based drug delivery device comprising wetted collagen particles of the present invention wherein the cells have been modified to express the desired drug.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a small molecule and or a large molecule drug.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a small molecule and or a large molecule drug wherein the drug is incorporated into the collagen particle before cross-linking.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a small molecule and or a large molecule drug wherein the drug is incorporated into the collagen particle after cross-linking.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and an antibiotic.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a growth factor.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a steroid.

Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a spermicidal agent.

Another embodiment of the invention is a drug delivery device comprising one or more drugs and the wetted particles of the present invention wherein each particle is encased in a complex coacervate gel.

Another embodiment of the invention is a drug delivery device comprising one or more drugs and micro-carrier composites comprising cells and the wetted, dehydrothermally cross-linked, collagen sponges of the present invention, wherein each sponge is encased in a complex coacervate gel

Another embodiment of the invention is a chromatography device

DETAILED DESCRIPTION OF INVENTION:

The present invention is directed to the development of sponges where sponge size, sponge shape, and pore size are maintained when the dry sponges are wetted into an aqueous medium. The sponges include particulates and sheets.

Accordingly, in one embodiment, the present invention relates to porous, particulate, wetted, dehydrothermally cross-linked collagen sponges wherein porosity is substantially maintained in the wetting process. Additionally, the invention is directed to methods of preparation of these sponges and methods of use thereof.

The term "sponge" is synonymous with the term "scaffold," and includes materials that provide a support structure, *e.g.*, for cells or in-growth of cells, and are suitable for containing a biological material, *e.g.*, a biological solution. The sponges of the present invention include non-spherical particulate, spherical particulate, and non-particulate sponges prepared by the methods described herein.

In one embodiment of the invention, the sponge is a biocompatible material, preferably a porous material, such as a porous biopolymer. In a preferred embodiment, the sponge comprises collagen, including anyone of the 21 types of collagen *e.g.*, types –I, -II, -III, –IV, etc. In one particular embodiment, the collagen is insoluble collagen. Soluble collagen may also be used. In a specific embodiment of the invention, the biopolymer is a cross-linked collagen, for example, bovine Type I collagen. Collagen for use in the sponges of the invention is commercially available, for example, from Sigma Aldrich in a variety of forms. Collagens from human, as well as animal sources, may be used, are commercially available. Recombinantly produced human and animal collagens, which are produced in a synthetic process by Fibrogen, may also be used. In addition, in one embodiment, the collagen may be extracted from animal tissue, *e.g.*, bovine or porcine tissues, *e.g.*, as described by Bell *et al.* in U.S. Patent No. 5,709,934.

Cross-linking may be induced chemically, thermally (*e.g.*, dehydrothermal cross-linking), or by radiation, *e.g.*, ultraviolet or gamma radiation. Cross-linking agents for chemical cross-linking include but are not limited to glutaraldehyde, formaldehyde and like

aldehydes; hexamethylene diisocyanate, tolylene diisocyanate, and like diisocyanates; ethyleneglycol diglycidylether, and like epoxides; and carbodiimide hydrochlorides. In a specific embodiment of the invention, the biopolymer is thermally cross-linked (*e.g.*, dehydrothermal cross-linking). In certain embodiments of the invention, the present invention does not use toxic cross-linking agents, *e.g.*, chemical cross-linking agents, like glutaraldehyde. In certain embodiments, the present invention does not utilize chemical modification.

In one embodiment, the sponges of the present invention are man-made or non-naturally occurring. This is distinguished from a naturally-occurring sponge from a human or animal source. Natural tissue comprises a collagen sponge and cells. A naturally occurring sponge is produced by de-cellularization of natural tissue leaving the collagen sponge, which retains the natural sponge-like properties. For the man made sponges the source of collagen may be animal or human, but the naturally occurring sponge is first reduced to an insoluble fiber or powder or a soluble solution of collagen. It is then reconstructed into a man made sponge.

In certain embodiments, the morphology of sponges of the present invention is unique. In one embodiment the distinction in the morphology of the sponges is the result of the source of the collagen used to prepare the sponge, *e.g.*, the collagen is commercially processed beyond the point that permits retention of natural sponge-like properties (*e.g.*, there is a loss of natural morphology), as opposed to derived directly from natural sources that allow retention of the natural sponge-like properties.

Wetted particulates of varying shape, particle size, and pore size have been produced using the methods presented herein. Spherical particles of 1μ to 10, 000μ, *e.g.*, 250μ to 2000μ, *e.g.*, 500μ to 2000μ, in diameter with wet maximum pore sizes from 5μ to 50μ were produced. Non-spherical particles of 0.5 to 2 mm in diameter (and larger) and wet maximum pore sizes from 5μ to 200μ were also produced. Smaller particles with smaller pores may be prepared from the methods of the invention. Particle sizes as low a 1μ may be made by the methods of the invention. Large spherical and non-spherical particle may also be made, *e.g.*, particles as big as 10,000μ, or even larger may be made by the methods of the invention.

A preferred process for producing dry sponges involves preparing a dispersion or solution of collagen in an aqueous, acidic medium, casting the aqueous mixture into the desired shape, freezing, and then lyophilizing. The pore size of the dry sponge depends upon freezing conditions, collagen concentration, and pH. More than other variables, freezing conditions affect the pore size of dry collagen sponges. In this regard, and without wishing to be bound by theory, pore size depends on the size of the ice crystals formed in the freezing step, and the size of the crystals is indirectly proportional to the freezing rate. If freezing is

performed isothermally in a liquid medium, pore size is proportional to the temperature of the freezing medium.

Freezing in liquid nitrogen results in very small pores *e.g.*, of about 5μ to10μ (for the largest pores). Freezing in liquid pentane, at about –15C, results in larger pores of up to about 40μ to 50μ. Much higher temperatures can not be used as the aqueous mixture will not freeze. However, if larger pores are desired, a gas may be used as the freezing medium (*i.e.*, at fixed temperature, heat transfer and freezing rate are slower in a gas than a liquid). Moreover, freezing in air at about –20C results in pores sizes up to about 200μ.

In a preferred embodiment, the spherical particulates of the present invention were produced by freezing in a liquid media and the largest pore sizes ranged from 5μ to 50μ. Although, it is contemplated by the invention to freeze spherical particulates in a gas at an appropriate temperature to yield larger pores. Non spherical particles are conveniently produced in processes where freezing may be done in a liquid or gas medium. Non- spherical particulates with maximum pore sizes ranging from 5u to 200u were produced.

Non spherical particulates are produced by preparing a dispersion or solution, casting into a shape that is much larger than the size of the desired particulate, freezing, milling, and lyophilization. In this regard, a cryogenic milling process can be utilized. Furthermore, particle size may be controlled by fractioning the frozen, ground dispersion, with a series of sieves in, for example, liquid nitrogen. However, other chilled liquids may also be used as the grinding and separation medium. In a particular embodiment of the invention, three fractions of particulate sizes are produced including one passing through a 5mm sieve and retained on a 2 mm sieve, a 2nd retained on a 0.5 mm sieve, and the remainder. However, other particle sizes are contemplated by the invention.

In certain embodiments, the cryogenic milling process and the separation of the particle sizes through the use of sieves may be performed simultaneously. Advantageously, higher yields of the desired particle fractions may be produced in comparison to the process that utilizes separate grinding and sieving steps.

Smaller dry particulates and processes to manufacture smaller dry particulates are contemplated. One option is to spray a solution or a dispersion directly into a liquid freezing bath. Another option is producing a "water in oil" emulsion, wherein the "water phase" is the solution or dispersion. The temperature of the emulsion is maintained below the freezing point of the dispersion or the solution. The frozen particulates made by either process are then lyophilized to produce the dry particulate sponges.

Maintaining Porosity Upon Wetting of Collagen Sponges:

In general, the lyophilized, dehydrothermally, cross-linked, sponges, *e.g.*, known sponges as well as sponges of the present invention, can be directly wetted with buffer or a biological solution, *e.g.*, a nutrient solution; however this causes shrinkage and reduction of pore size. More specifically, the wetting process of the invention is intended to be useful for all sponges, regardless of their method of preparation. For example, in addition to preparation by the methods of the present invention, sponges that may benefit from the wetting processes described herein may be prepared from solutions that are directly dehydrated using heat and or vacuum to produce the sponge morphology, which may then be dehydrothermally cross-linked.

The language "biological solution" comprises a biological material, *e.g.*, cells, contained in a liquid medium, *e.g.*, aqueous solutions, *e.g.*, water or buffered aqueous solutions. In one embodiment, the biological solution is a nutrient solution supportive of cell growth. However, it should also be noted that the biological material may be the liquid medium, for example, water or buffered solutions.

The language "biological material" includes a material or agent that is biocompatible with a subject, *e.g.*, an animal, *e.g.*, a human. Examples of biological materials include, but are not limited to water, buffered solutions, saline, nutrient solutions supportive of cell growth, cells, cell cultures, proteins, amino acids, cytokines, *e.g.*, lymphokines, blood products, hormones, antibodies, *e.g.*, monoclonal, toxins, toxoids, vaccines, *e.g.*, viral, bacterial, endogenous and adventitious viruses, and pharmaceutical agents; *e.g.*, pharmaceutical drugs. In one embodiment of the invention, the biological material is a biological solution.

The language "nutrient solution supportive of cell growth" includes solutions that contain nutrients, *e.g.*, amino acids or growth factors supportive of cell growth. Optionally, the nutrient solution can contain cells.

In one embodiment, the invention is directed to a stepwise method for the retention of porosity upon wetting a dehydrothermally cross-linked collagen sponge with an aqueous medium. This can be best appreciated from an examination of the confocal microscopy images in Fig 56 for sample nos. 1, 4, and 7 described in the Overview of the Exemplification. Comparison of the porosity for samples wetted *via* the nine step process (described in Example 3 and the Overview of the Exemplification) may easily be made to that for samples wetted directly in PBS. Samples 1 and 4 are porous when wetted *via* the nine step process, and pore size is similar to that for the dry samples in Fig 54. When samples 1 and 4 are wetted directly in PBS, the porosity is totally collapsed. Sample 7 comprises much larger pores than samples 1 and 4. Sample 7 is porous when wetted *via* the nine step process and porosity is similar to that for the dry sample. When sample 7 is wetted directly in PBS

there is some collapse of porosity versus the nine step method, but the reduction in porosity is not as dramatic as that for the smaller pore size samples, 1 and 4.

Particle size measurements complement confocal microscopy results. Moreover, reduction of particle size upon wetting is an indirect method of measuring reduction of porosity. The order of porosity reduction upon wetting, as measured *via* change in particle diameter or particle cross-sectional area is as follows: direct in PBS (32% to 67%) > direct in 70% ethanol (41%) >> 2 step (-0.2% to 7.6%) >/= 9 step process (-1.6% to 6%).

The values for particle size reduction after wetting, cited above, are a compilation of values for sample nos. 1, and 7 in Table 1. Intermediate behavior is expected for Sample no. 4 because of its intermediate pore size. Wetting directly in PBS or 70% ethanol results in a significant decrease in porosity, and wetting *via* the stepwise processes results in retention of porosity after wetting.

The mechanism of porosity retention for the new wetting procedures deserves some attention. Without wishing to be bound by theory, surface tension of the wetting agent likely plays a role. It may be difficult to wet a dry collagen particle with a high surface tension liquid such as water or PBS. Instead of filling the pores in the interior of the sponge, which are initially filled with air and or water vapor, the liquid crushes the sponge. The force required for the liquid to penetrate the pores exceeds the compressive strength of the dry sponge. The surface tension of water is 75 dynes/cm^2, and that for ethanol is 22 dynes/cm^2 . Ethanol can penetrate the pore without collapsing the structure. Once the pores are filled with liquid, the structure is not crushed by addition of a higher surface tension liquid to the liquid particle slurry.

Although one would expect that wetting directly in a high alcohol, water/alcohol mixture would also preserve porosity effects, a 70% alcohol / 30% water solution, which has a surface tension of 26.3 dynes/cm2 (just slightly higher than for ethanol alone) resulted in a significant decrease in porosity upon direct addition. Therefore, the compressive strength of the dry sponge may be less than 26.3 dynes/cm^2.

Furthermore, pore size for the dry sponges should play a role. Reduction of porosity upon wetting should be indirectly proportional to pore size, based on the surface tension explanation. This is apparent from measurement of particle size reduction upon wetting. The sponge with the smallest dry pores, 5μ to 10μ, exhibits the largest reduction, 67%, of porosity upon wetting directly into PBS. The sponge with the largest pores, ~200μ, exhibits a reduction of 32% upon wetting. Both values are measurements of the reduction in maximum particle diameter upon wetting. A 57% reduction of in cross-sectional area is also reported for the large pore sample, but the change in cross-sectional area may be expected to be more dramatic than that for maximum particle diameter. Moreover, results based on the confocal images are consistent with particle measurements.

In addition, collagen concentration is expected to have an effect on porosity reduction upon wetting.

In another embodiment, variations of the step wetting procedure are contemplated. The 1st step involves wetting dry sponges into a low surface tension, water soluble liquid. Tranformation to an aqueous medium may be accomplished in a continuous process or semi-continuous process, instead of a batch process. Aqueous mixtures may be caused to flow through sponges wetted with the non-aqueous, water soluble solvent.

In certain embodiments, the non-aqueous solvent is ethanol, isopropanol, methanol, acetone, dimethyl ether, other water soluble alcohols and ketones. In a specific embodiment, the non-aqueous solvent is ethanol.

It should be noted that both the process of preparation of the wetted sponges and the sponges prepared from the wetting process, including further preparations that use the wetted sponges, *e.g.*, composites, described herein are contemplated by the present invention. For example, one embodiment of the invention is a process for wetting sponges with a sequence of five wetting agents and the sequence of five wetting agents comprises:

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 65% non-aqueous, water soluble solvent/6% to 35% water;

64% to 35% non-aqueous, water soluble solvent/ 36% to 65% water;

34% to 6% non-aqueous, water soluble solvent/ 66% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95% water,

as well as the wetted sponges and composites made therefrom.

In an additional embodiment, the invention is directed to a process for wetting sponges with a sequence of four wetting agents and the sequence of four wetting agents comprises

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 50% non-aqueous, water soluble solvent/6% to 50% water;

49% to 6% non-aqueous, water soluble solvent/ 51% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95%,

as well as the wetted sponges and composites made therefrom.

Another embodiment of the invention is a process for wetting sponges with a sequence of two wetting agents and the sequence of two wetting agents comprises:

100% to 95% non-aqueous, soluble solvent; and water, as well as the wetted sponges and composites made therefrom.

Applications of Sponges of the Invention

The sponges of the present invention may be used for any application that could make use of the support structures of the invention. In certain embodiments, the sponges of the present invention may be used in tissue composites, as resorbable carriers of biological materials including pharmaceuticals, or in chromatography devices.

A) Tissue Composites Prepared From Collagen Sponges of the Invention

In one embodiment, the invention is directed to improved tissue composites, *e.g.*, biocompatible composites, prepared from the sponges of the invention, which overcome or minimize the problems associated with existing tissue repair systems and can be easily prepared and maintained in a sufficient quantity, and suitable shapes, to enable convenient treatment of tissues requiring repair. Further discussion of the methods of preparation of these tissue composites is contained in PCT Application Number PCT/US03/10439, which is hereby incorporated herein by reference.

As used herein, the term "composite" includes a substantially solid material that is composed of two or more discrete materials each of which retains its identity, *e.g.*, physical characteristics, while contributing desirable properties to the composite. For example, in certain embodiments of the invention, the composite is produced by two biopolymers each having independent physical characteristics, *e.g.*, degree of cross-linking or porosity. Composites of the invention typically include a biocompatible scaffold or sponge of the present invention and a biocompatible gel.

As used herein, the term "gel" includes materials that exist in a two-phase colloidal system consisting of a solid and a liquid in more solid form than liquid form, *i.e.*, a semi-solid, of low porosity capable of retaining or immobilizing cells, while allowing the cells to proliferate. Accordingly, the gel is preferably formulated to allow diffusion of nutrients and waste products to, and away from cells, which promotes tissue growth following contact of a subject with a composite. In addition, the gel is preferably formulated to provide structural support to components of the composite, *e.g.*, cells, during formation of the composite. The terms gel is intended to include materials that function as a "glue" to retain components of the

composite in their desired location during formation of the composite as well as maintain the structural integrity of the composite following preparation and initial implantation in a subject. This aspect is particularly advantageous for composites in which the scaffold comprises particulates.

Preferred materials for use in composites of the invention are biopolymers. As used herein, the term "biopolymer" includes biocompatible materials composed of one or more polymeric materials that are typically formed in a biological system or synthetically prepared from biologically available monomers. A biopolymer of the invention can be in the form of a solid, semi-solid, or liquid, and can be isolated from a biological system or synthetically prepared. Additionally, biopolymeric solidification of a solution can occur, *e.g.*, by aggregation, coagulation, coacervation, precipitation, ionic interactions, hydrophobic interactions, or cross-linking. In one embodiment of the invention, the biopolymer is a cross-linked biopolymer. Cross-linking of the materials of the composite may be induced chemically, thermally (*e.g.*, dehydrothermal cross-linking), or by radiation, *e.g.*, ultraviolet. Cross-linking agents for chemical cross-linking include but are not limited to glutaraldehyde, formaldehyde and like aldehydes; hexamethylene diisocyanate, tolylene diisocyanate, and like diisocyanates; ethyleneglycol diglycidylether, and like epoxides; and carbodiimide hydrochlorides. In a specific embodiment of the invention, the biopolymer is thermally cross-linked (*e.g.*, dehydrothermal cross-linking).

For use *in vivo*, particularly in human subjects, it is preferred that the materials that compose the composite include materials that are biocompatible with the subject. The term "biocompatible" includes materials that are compatible with a subject and are not toxic or deleterious to the subject. In certain embodiments of the invention, the biocompatible material is biodegradable, such that it degrades or decomposes following contact with a subject, *e.g.*, human.

In specific embodiment, the biocompatible material is a biopolymer. Examples of commercially available biocompatible materials include collagen, *e.g.*, types I to XX1 including –I, -II, -III, and -IV, gelatin, alginate, agarose, *e.g.*, type –VII, carrageenans, glycosaminoglycans, proteoglycans, polyethylene oxide, poly-L-lactic acid, poly-glycolic acid, polycaprolactone, polyhydroxybutarate, polyanhydrides, fibronectin, laminin, hyaluronic acid, chitin, chitosan, EHS mouse tumor solubilized extract, and copolymers of the above. However, the specific use of non-resorbable polymeric components, or of non-polymeric resorbable components such as soluble bioglasses is not precluded.

Alternative biopolymers for use in the composites of the invention include complex coacervates. The term "complex coacervate" includes an aggregate, *e.g.*, of colloidal droplets, held together by electrostatic attractive forces. Additionally, the aggregate may be hydrated, *i.e.*, comprising water. In certain embodiments of the invention, the complex

coacervate comprises calcium alginate and gelatin A, or calcium alginate. In one embodiment of the invention, a complex coacervate gel is prepared by contacting a biocompatible porous scaffold comprising a first component of the complex coacervate, *e.g.*, calcium alginate, with a biopolymer solution comprising a second component, *e.g.*, gelatin A, of the complex coacervate. The combination of the two components upon the combination of the scaffold with the biopolymer solution acts to solidify the biopolymer solution through coacervation and aggregation.

Additionally, other biopolymers for use in the composite include agarose and mixtures of agarose and gelatin A. Preferably, the melting point for a gel comprising agarose and gelatin A is lower than for a gel comprising agarose alone. In a specific embodiment, the agarose mixture is a low temperature melting agarose.

The term "alginate" includes the salt or ester of an insoluble colloidal acid $(C_6H_8O_6)_n$, which in the form of its salts is a constituent of the cell walls of brown algae. In certain embodiments, the alginate exists as a calcium salt, and is thus termed a calcium alginate. Alginate is a polysaccharide, which can be derived from brown seaweeds, composed of D-mannuronic and L- glucuronic acid monosaccharide subunits. While the sodium salt of alginate forms viscous solutions, alginate can form hydrated gels in the presence of divalent cations such as calcium due to cross-linking through the negatively charged carboxyl groups residing on the L-glucuronic acid residues. The viscosity of the uncross-linked solutions and thereby the mechanical strength of cross-linked gels can be influenced by altering the average chain length of the alginate or by altering the proportion of D-mannuronic acid and L-glucuronic acid residues within the polysaccharide. These factors may also influence the rate of resorption of the alginate. Alginate is commercially available, for example, from Kelco International Ltd. Waterfield, Tadworth, Surrey, UK.

The term "gelatin" includes a variety of substances (such as agar) resembling gelatin, *e.g.*, glutinous material obtained from animal tissues by boiling, *e.g.*, colloidal protein used as a food, in the art of photography, and in the art of medicine. Gelatin A is prepared by briefly treating pigskins with dilute acid followed by extraction with water at 50-100 °C. The resulting gelatin A has a high isoelectric point (pI), and thus is positively charged at physiological pH.

The term "agarose" includes a polysaccharide obtained from agar, *e.g.*, known in the art as a common supporting medium in gel electrophoresis. Agarose is commercially available, for example, from Sigma, Poole, England.

The term "gelling," is well known in the art, and includes the act of becoming solid or thickened by chemical or physical alteration, thereby changing into a gel.

In another embodiment, the invention is directed to a multi-cellular composite comprising at least one first multi-functional unit (MFU), and at least one second MFU. In this embodiment, the multi-cellular composite contains at least one MFU that comprises a first biocompatible porous scaffold in contact with a first biocompatible gel seeded with a first population of cells wherein the gel is in contact with at least one surface of the scaffold.

The language "multi-cellular composite" includes composites of two or more cell populations. In preferred embodiments of the invention, at least one of the two or more cell populations is seeded in gel in desired compartments in the composite such that the cell types are located to provide a specific tissue function in a subject. For example, in one embodiment of the invention, the first population of cells comprises fibroblasts and the second population of cells comprises keratinocytes.

Other embodiments of the invention involve the preparation of tissue composites of different shapes or forms using composites of the invention. The composite can be shaped to corresponded to the desired tissue to be formed, *e.g.*, soft tissue, *e.g.*, skin, bone, an organ, *e.g.*, cartilaginous tissue, *e.g.*, a meniscus for a knee, an ear, a nose, or other tissue. The shape of the composite may be equally affected by the shape of the individual components of the composite, *i.e.*, the scaffold or the gel. Molding the composite to the desired shape can be achieved by selecting the shape of either the scaffold or the gel. In one embodiment, the shape of the composite is a product of a mold in which either the scaffold or the gel or both the scaffold and the gel are formed. For example, after mixing the desired cell types, the gelling agent and the collagen scaffold at a condition that will retard the gelling of the mixture, the mixture can be injected or cast into a mold of the desired structure under appropriate conditions to facilitate gelling of the mixture to the desired structure.

In another embodiment of the invention, a composite is prepared on the surface of a mesh to facilitate transfer to a subject. Preferred mesh comprises a polymer that is not bioabsorbable, preferably having a pore size ranging from 3 to 216 microns in diameter. In one embodiment, a nylon mesh is be used to reduce shrinkage of the composite, particularly with composites containing fibroblasts. It has been determined that shrinkage of the composite during *in vitro* culture is analogous to wound contraction *in vivo*, and therefore, the mesh and the desired size of the collagen particulates in the composite may be used advantageously in reducing wound contraction, if any, *in vivo*. Additionally, the mesh may be used to assist in handling of the composite prior to implantation in a subject or to assist in forming the composite into a desired shape.

A composite or sponge of the invention may be affixed to the patient through grafting techniques known in the art, for example, such as described by J. Hansbrough *et al.* (Journal of Med. Assoc., vol. 262, No. 15, Oct. 20, 1989 pp. 2125-2130. J. Hansbrough, S. Boyce, M. Cooper, T. Foreman Burn Wound Closure With cultured Autologous Keratinocytes and

Fibroblasts Attached to a Collagen-Glycosaminoglycan Substrate). Additionally, the composite may be affixed to the subject through gelatinization, or lamination, as described by Morota *et al.* in U.S. Patent No. 6,051,425.

For use in tissue repair, composites of the invention include one or more cell populations. Typically, the composite is seeded with cells of at least one cell type. The language "seeded with cells" includes a distribution of cells retained or immobilized within a material that contributes to the composite, *e.g.*, the gel or scaffold. In certain embodiments, the distribution of cells is retained or immobilized in, for example, the gel, the scaffold, or both. The distribution of cells may be of a single type or of multiple types, *e.g.*, as in the multi-cellular composites. In certain embodiments of the invention, the distribution of cells is a uniform distribution. In an embodiment where both the scaffold and the gel are seeded with cells, the cells may be selected for a specialized function *in vivo* (*e.g.*, dermal and epidermal cells for skin repair) or be seeded with cells for independent function. Cells are selected and added to the material such that the composite can perform its intended function. Cells for use in the composites can be primary cells harvested from a donor, cultured cells, *e.g.*, allowed to proliferate *in vitro*, or cryopreserved cells. Acellular composites may also be produced using the appropriate methods of the invention.

The language "cells contained in," for example, in the expression, "the cells contained in the scaffold," refers to a dispersion of cells in a biocompatible material, *e.g.*, biopolymer, or adsorption of the cells and/or cell solution onto the surfaces of a biocompatible material. In contrast, the language "seeded with cells," refers to retention, or immobilization, and placement of cells within a biological material.

Cell types for use in the methods and compositions of invention include, for example, fibroblasts, keratinocytes, and stem cells. Cells for use in the methods and compositions of invention include primary cells, cultured cells and cryopreserved cells.

Examples of cell types for use in the methods and compositions of invention include but are not limited to epidermal and dermal cells (*e.g.*, keratinocytes or fibroblasts), muscle cells (*e.g.*, monocytes), cartilage cells (*e.g.*, chondrocytes), bone forming cells (*e.g.*, osteoblasts), epithelial cells (*e.g.*, corneal cells, tracheal cells, or mucosal cells), endothelial cells, pleural cells, ear canal cells, tympanic membrane cells, peritoneal cells, gingiva cells, neural cells, hepatocytes, pancreatic cells, cardiac cells, and stem cells.

Cells for use in the methods and compositions of invention can be isolated from a tissue biopsy or bone marrow sample from a subject, using methods known to those skilled in the art. If insufficient cell numbers are available at isolation, the cells can be allowed to proliferate in culture prior to seeding into a composite of the invention. During cell growth and proliferation, the cells can be cultured as a monolayer on a tissue culture treated substrate and maintained in tissue culture medium such as Dulbeccos Modified Eagle's Medium

supplemented with, for example, between 1 and 20% fetal calf serum or autologous human serum. Alternatively, the cells can be cultured in serum free medium supplemented with mitogens on tissue culture plastic modified by the immobilization of specific attachment factors. In another approach, isolated cells can be seeded at a specified seeding density within alginate beads and cultured in tissue culture medium supplemented with serum or mitogenic growth factors. The cells can be isolated by dissolving the beads in a sodium citrate saline solution followed by collagenase digestion. The cells can be cultured within a suitable bioreactor.

In a particular embodiment for skin repair, cells are obtained from skin sample from a subject to be treated (autologous) or from donor tissue (allogenic). Skin samples are treated with trypsin to separate the epidermis from the dermis (Eisinger, M. Method in Skin Research, Editor D. Skerrow, (1985) pp 193). The epidermis is minced and treated with trypsin to release keratinocytes. The keratinocytes are then cultured until confluence using standard methods. In certain embodiments, the keratinocyte cells are cultured as single cell suspensions until confluence. Alternatively, in a preferred embodiment, the keratinocyte cells are seeded as single cell suspensions and cultured until confluence.

Primary cultures of fibroblast cells for use in accordance with the present invention may be prepared using standard methods such as, for example, the method disclosed in "A specific collagenase from Rabbit fibroblasts in monolayer culture," Journal of Biochemistry (1974) 137, 373-385. Preferably, primary cultures of fibroblasts are prepared as follows. A dermal sample is cut up into 1 mm cubes and is suspended in a solution of collagenase buffered with Tris-HCl pH 7.4. A suitable collagenase is *Clostridium histolyticum* collagenase. The dermal sample is preferably suspended in solution at a concentration of 1 microgram/mL. The suspension is incubated and then centrifuged at 1,500 rev/sec to remove the cells from solution. The suspension is preferably incubated for 30 minutes. The cell pellet is washed with DMEM and the number of fibroblasts is determined with a haemocytometer. The viability of the fibroblast is determined by dye exclusion using Trypan Blue. The above culturing method also surprisingly yields other dermal epithelial cells that have a potential to develop into sweat glands or other skin cell types. An additional source of fibroblasts and keratinocytes includes neonatal foreskin, in which the cells can be isolated by standard protocols as described above.

B) Delivery Devices Prepared From Collagen Sponges of the Invention

In one embodiment, the invention is directed to an enclosure comprising the wetted spherical and/or non-spherical particulates, of the present invention. An enclosure is a mold capable of receiving the sponges of the present invention. In certain embodiments, an enclosure is a device capable of containing a composition, such that the device becomes at

least an integrated component of the resulting composition, *i.e.*, a composite prepared in a mold containing a mesh anchoring portion, or a wound sealed at the exposed surface with a film or fabric or some other suitable cover that encloses the wound and becomes integrated with the final composition. In specific embodiments, the enclosure is the film or fabric, *e.g.*, porous fabric, or some other suitable cover that contacts the composition, *e.g.*, the sponges of the present invention. The enclosure and enclosed composition may be an engineered tissue composition and or a carrier device, *e.g.*, a drug delivery device. In certain embodiments, at least one face of the enclosure is living tissue.

The shape of the enclosure or mold is tailored for the end use. For example, the shape could be an element of tissue to be replaced/regenerated. Compositions comprising the wetted spherical and/or non-spherical particulates are cast into the mold. If an engineered tissue is to be constructed, the mold and contents may be cultured in a nutrient medium, *e.g.*, *in vitro* or *in vivo*.

Another embodiment of an enclosure is a "mold" containing wetted spherical and/or non-spherical particulates, cells, and a "vascular system" that supplies a flow of nutrients. The vascular system may be designed to mimic that in a human or animal. A further embodiment of this invention is the use of particulates seeded with cells. The seeded particulates are cultured in a bioreactor to produce seeded particulates with a high cell density. These are placed in the enclosure comprising the vascular system and cultured in vitro or in vivo. Advantageously, this embodiment overcomes the problems associated with the delivery of nutrients to thick sections of engineered tissue.

In another embodiment, the invention is an enclosure comprises a carrier device comprising the wetted spherical and/or non-spherical particulates, of the present invention and an additional component. In certain embodiments, the additional component is a microorganism, *e.g.*, bacteria, cells, *e.g.*, a drug, pharmaceutical agents, *e.g.*, small and large molecules, cells modified to express a desired pharmaceutical agent ,antibiotic, growth factor, steroid, spermicidal agent, and the like, as well as combinations thereof. Accordingly, the carrier devices may be comprises of solely the sponges and the additional agents or may be comprised of sponges as part of a composite (also referred to as micro-carrier composites. The carrier devices of the invention may be cellular, *e.g.*, a cell-based drug delivery device, or acelluar.

In certain embodiments, each particle of the carrier is encased in a complex coacervate gel. It should be noted that the process of preparing such complex coacervates, as described herein, may be used to coat medical devices, *e.g.*, stents, which are to be implanted into a subject, and such an application is within the scope of this invention.

The additional component of the carrier device may be incorporated into the collagen particle before or after cross-linking, *e.g.*, addition of the additional component may occur at

the dispersion stage or after dehydrothermally cross-linking.

Another embodiment of the invention is an aqueous dispersion or slurry comprising the spherical and/or non-spherical particulates, of the present invention, and a microorganism.

Yet another embodiment of the invention is a medical sealant comprising an aqueous dispersion or slurry comprising the spherical and/or non-spherical particulates, of the present invention.

C) Chromatography Devices Prepared From Collagen Sponges of the Invention

In another embodiment, the invention is a chromatography media comprising the wetted spherical and/or non-spherical particulates of the present invention. Chromatography devices of the invention may be monolithic in nature or may be composed of packed particles, which are useful for chromatographic separations, *e.g.*, size exclusion or affinity. In certain embodiments, the sponges of the present invention may also be useful as a filter media.

Another embodiment of the invention is a device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids. The container may be any receptacle capable of holding the sponges of the invention, *e.g.*, both particulate and non-particulate (*e.g.*, sheets, *e.g.*, producing the monolithic chromatographic medium), which would be acceptable for use in the chromatographic arts, *e.g.*, glass or steel. The polymer may be a naturally occurring biopolymer, *e.g.*, a protein, polysaccharide, or lipid, which may also be cross-linked, *e.g.*, dehydrothermally cross-linked, chemically cross-linked, or cross-linked by radiation. In specific embodiments of the chromatographic device, the biopolymer is collagen. The polymer may be water-swellable.

In one embodiment, the invention is directed to a method of producing device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids comprising the following steps:

producing an aqueous solution and/or a dispersion of a polymer;

filling a tube with the solution and/or dispersion of a polymer;

freezing the solution and/or dispersion of a polymer in the container; and

lyophilizing the container filled with the frozen solution and or dispersion of a polymer.

In certain embodiments, the aqueous solution or dispersion further comprises an organic

solvent. In additional embodiments, the aqueous solution or dispersion is frozen in a bath, e.g., liquid nitrogen, maintained at a temperature below the freezing point of the solution and or dispersion of the polymer.

Another embodiment of the invention is a method of producing a device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids comprising the following steps:

producing an aqueous solution and/or a dispersion of a polymer;

freezing the solution and or dispersion of a polymer in the shape of the container;

lyophilizing the shaped, frozen solution and/or dispersion of a polymer to form a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids into a container.

inserting the shaped, lyophilized, monolithic interpenetrating network into the container

sealing the monolith into the container to insure that the monolith is in contact with interior wall of the container.

Additionally, the contact between the monolith and the container wall may be established by hydrating the lyophilized monolith inside the tube. In particular embodiments, the lyophilized monolith is further subjected to the steps of:

wetting in a non-aqueous water soluble solvent and then

exposing the wetted cross-linked scaffold to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water.

Definitions

In addition, several additional terms have been used in and throughout the specification; for convenience the definitions of these terms are shown below:

The term "casting" is well known in the art, and includes the process by which a material is formed into a shape to by pouring liquid into a mold and letting harden without pressure. In one embodiment of the invention, the hardening of the material is performed through temperature changes. In another embodiment of the invention hardening of the material is performed *via* complex coacervation. In certain embodiments of the invention, the casting of the scaffold is accomplished by exposure to low temperatures, *e.g.*, liquid nitrogen.

The language "contact" or "contacting" includes the union or junction of surfaces. The union may be made through a single point, in a region, *i.e.*, surface, or in separate points or separate regions. The term "surface" as used herein includes the outer periphery, exterior, or upper boundary of a material. In certain embodiments, the term surface is used herein to describe a sheet structure, *e.g.*, a scaffold in the form of a sheet, which is generally planar, *e.g.*, a planar or curved, two-dimensional locus of points (as in the boundary of a three-dimensional region). In certain embodiments, contact of one surface is made with a primary face, *e.g.*, a first primary face, of another surface. The language "primary face" includes surfaces of sheet structures that are comparatively larger than other surfaces of the sheet structure. Several examples of materials in contact are shown in Figures 1-5 of PCT Application PCT/US03/10439.

The term "continuous web process" is one in which a liquid or liquid-like material is coated on to a web (film, paper, foil, or fabric) in a continuous process. In one embodiment, a liquid is the ungelled mixture of cells, gellable solution, and particulate sponges, and the web is a porous nylon fabric; gellation occurs after coating as a result of a change in temperature.

The language "improving a condition of a tissue" includes growth of new tissue, protection of the tissue, *e.g.*, from injury, *e.g.*, infection, prevention of fluid loss, and tissue support to improve conditions for natural repair mechanisms of the subject. In one embodiment, contacting the tissue of a subject with a composite of the invention returns the tissue to a healthy state.

The language "multi-functional unit (MFU)" is intended to include distinct geographical and functional units (*e.g.*, a unit with a distinct biological activity/function, *e.g.*, a unit distinctly positioned for the growth of separate populations of cells) of a multi-cellular composite, wherein each functional unit may comprise a gel, a scaffold, a biological material, *e.g.*, a cell population, or any combination thereof. For example, in certain embodiments of the invention, scaffold and gel combine to form one distinct multi-functional unit of a multi-cellular composite. In certain other embodiments, scaffold, gel, and cells are combined to

form a single multi-functional unit. It should be understood that the inclusion of a biological material in a single MFU is not limited to a single biological material, *e.g.*, a single MFU may contain more than one type of cell in a cell population.

The term "particulate" as defined herein, includes materials, *e.g.*, biopolymers, which are particle in nature, *e.g.*, relatively minute, small, or discrete. In the present invention, the term "particulate" is intended to include both spherical and non-spherical particulates.

The term population" includes a group of individual objects, or items from which samples are taken for statistical measurement.

The term "porous" includes materials having pores through which substances can pass. In certain embodiments of the invention, the scaffold component of the composite has an average pore size that allows for cell growth, for example, a porosity that allows nutrients and waste products to diffuse through the material. In another embodiment, the sponge has an average pore size that allows for the in-growth of cells.

The language "subject" includes animals *e.g.*, mammals, *e.g.*, dogs, cats, horses, pigs, cows, sheep, goats, rodents, mice, rats, rabbits, squirrels, bears, and primates *e.g.*, chimpanzees, gorillas, and humans, as well as transgenic non-human animals. Preferably, the subject is a human, *e.g.*, a human requiring treatment of a tissue, *e.g.*, wound repair.

The language "surface porosity" refers to the total surface area of the pores on the surface of the sponge, *i.e.*, the pores that immediately accessible to the a biological material that would be added to the sponge, *e.g.*, an aqueous solution.

The term "tissue" includes cellular material capable of forming a collective entity. In one embodiment, a tissue is a collection or aggregation of morphologically and functionally similar cells. The term "wound" includes bodily injuries, including those which result in injury to a tissue, *e.g.*, skin, *e.g.*, a dermal wound.

The terms "treating" and "treating a tissue or wound" are intended to include improving at least one condition of a tissue or wound, and tissue augmentation, *i.e.*, plastic surgery, *e.g.*, lip injections of composites.

The language "volume fraction" of component, is defined as:

$$\frac{\text{Volume of component}}{\text{Total Volume of composition}}$$

Accordingly, the volume fraction of a component is a number between 0 and 1.

The term "washing" is related to the term wetting, and includes the process of wetting a material with a liquid that has already been already bee made wet, *e.g.*, to replace a non-aqueous water soluble solvent with an aqueous medium.

The term "wetting," is well known in the art, and includes the act of making a material wet. For example, in one embodiment of the invention involves the wetting of a

biocompatible porous scaffold with a biological material, *e.g.*, a biological solution. In addition, the wetting (or washing) may be performed in a batch or continuous process.

EXEMPLIFICATION

General Overview of Exemplification

Insoluble type I bovine collagen from SIGMA was used for most formulations. One formulation is prepared with collagen from a human source supplied by Sigma and another formulation is prepared with recombinantly produced collagen from Fibrogen. Collagen, acetic acid, and water were mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture was stored overnight in a cooler above the freezing point of the dispersion. See Examples below for specific formulations.

To prepare dry spherical collagen sponges, a collagen dispersion was metered with a peristaltic pump through a vibrating no. 22 needle dropwise into a bath of liquid nitrogen. Frozen specimens were lyophilized for 5 days at a pressure < 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days. In one embodiment, spherical sponges were prepared by casting droplets into a pentane bath at –15C.

In addition, in the methods for the preparations of the sponges, collagen concentration was varied from 1 mg/ml to 10 mg/ml, and acid concentration was varied from 0.5% to 0 5% by weight. Formulations comprising high collagen and or low acid concentrations could not be pumped through the needle due to high viscosity and/or large particle size. At low collagen concentration and low acid concentration significant deformation occurred upon lyophilization and the particles were not spherical.

In certain embodiments, optimum collagen and acid concentrations for dry sphere production, in liquid nitrogen or pentane, are 5 mg/ml and 5% by weight, respectively. The sponges exhibited a highly porous open cell structure. Spheres comprising this formulation, cast in both liquid nitrogen and pentane at –15C were used in wetting experiment described below.

To prepare dry non-spherical particulates, a collagen dispersion was poured into ice cube trays. The trays containing the dispersion were placed in a foam polystyrene container with a lid. The whole assembly was placed in a freezer set to –20C. The intent was to have slow cooling to generate a large pore size. The dispersion was chilled for, at least, 2 days at which point the dispersion is frozen.

Frozen cubes were quickly removed from the cooler, split in half, and added to a stainless steel sieve suspended in a liquid nitrogen bath. The sieve was agitated with a shaker. The cubes, immersed in liquid nitrogen, were ground with a high speed kitchen type mixer, such that ground particles smaller than the sieve fall through. The ground frozen particles may be separated into additional fractions with additional sieves. Frozen particle fractions

were lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days. Collagen concentration was varied between 5 mg/ml and 50 mg/ml and acid concentration was varied from 0.5% to 5% by weight.

Formulations comprising 50 mg/ml collagen were extremely viscous, and frozen, lyophilized and dehydrothermally cross-linked materials prepared from these dispersions were either non-porous or exhibit closed cell structures. Open cell sponges were produced with a dispersion comprising 5 mg/ml collagen and 5% acid. These are used for wetting experiments described below.

Other variations of non-spherical particles may also be prepared. In one embodiment, the non-spherical particles were made by casting large droplets of collagen dispersion into liquid nitrogen. In another embodiment, the non-spherical particles were made by casting and freezing in ice cube trays at –80C. In addition, preparation conditions for the non-spherical particles may be the same as described above for spherical particles.

Dry sponges were imaged with SEM. Representative SEM images of dry particles are depicted in Figure 54 for sample nos. 1, 4, and 7 in Table 1. Maximum pore size was estimated visually. Average pore size was measured with Image Pro Plus 4.5 and the dry pore size measurements are reported in Table 1.

Dry sponges may be wetted by various procedures. One embodiment involves wetting with the series of nine ethanol-phosphate buffer (PBS) mixtures shown below as 1 through 9. The collagen sponges were first added to a flask containing absolute ethanol. Reduced pressure was applied for a short duration to facilitate wetting. The flask was sealed and shaken until the particles sank. Most of the ethanol was decanted off and the second alcohol / PBS mixture was added. The flask was shaken again until the particles sink. This procedure was repeated for the remaining alcohol / PBS mixtures.

1. 100% ethanol
2. 85% ethanol / 15 % PBS
3. 75% ethanol / 25% PBS
4. 65% ethanol / 35% PBS
5. 60% ethanol / 40% PBS
6. 50% ethanol / 50% PBS
7. 30% ethanol / 70% PBS
8. 15% ethanol / 85% PBS
9. 5% ethanol / 95% PBS

For a 2 step process collagen sponges were first added to a flask containing absolute ethanol. Reduced pressure was applied for a short duration to facilitate wetting. The flask was sealed and shaken until the particles sank. Most of the ethanol was decanted off and PBS was added. The flask was shaken again until the particles sank.

Collagen sponges may also be wetted directly into either PBS or 70% ethanol in water. Reduced pressure was applied for a short duration to facilitate wetting. The flask was sealed and shaken until the particles sank.

Measurement of particle size before and after wetting is an indirect method of retention of porosity upon wetting. Figure 55 presents a comparison of particle size reduction for two different wetting procedures. Measurements were made for various particles wetted by different methods (See Table 1). Maximum particle diameters were measured for a population of 10 to 20 particles prior to wetting and after wetting with a stereo microscope fitted with a graded eyepiece. Similar measurements may be made with an image analysis tool, Image Pro Plus. Both maximum particle diameter and cross-sectional area were measured. The percentage change in maximum diameter and cross-sectional area is reported in Table 1 for various particles and wetting procedures.

Porosity after wetting was also evaluated with confocal laser scanning microscopy. Sponges were stained with Alexa Fluor 488 carboxylic acid dye solution 1mg/ml PBS (Molecular Probe Cat # A-20000). A Zeiss LSM400 microscope was used and the emission at 488 was observed. Images of the 3 different particles wetted *via* the nine step process and directly into PBS are shown in Figure 56.

Images of a sheet sponge, frozen at –20C, wetted *via* the two step process and by the nine step process are shown in Figure 57.

To ascertain the ability of these particles to support cell growth in vitro, an equivalent volume of each type of collagen particles, labeled sample nos. 1, 2, and 3, were used. Porcine fibroblasts, (3×10^6) were mixed with the washed particles in a 6-well plate insert with a 0.4 micron mesh at the bottom in a 100-mm petri dish. The cells and particles in the insert were incubated in 2 ml of F12/DMEM medium containing 15% fetal calf serum, supplements and antibiotics at 37 C in a CO_2 incubator for two hours. The whole insert was subsequently covered with culture medium and further incubated at 37 C for the duration indicated. Alternatively, the collagen particles with the cells were transferred to a spinner flask after overnight incubation at 37 C in a 100-mm dish.

The proliferation of the fibroblasts was determined by confocal microscopy. As indicated in Figure 58, all three types of particles support the proliferation of the cells. In particular, higher cell density is observed from cultures (Figures 58B and 58C) incubated for

10 days in spinner flasks when compared to that (Figure 58A) incubated for 6 days in a 100 mm Petri dish. Accordingly, the degree of proliferation depends on the duration of the incubation as well as the type of culture vessels used for the study.

No.	Dry Particulate Shape	Casting Medium	Casting Temperature (F)	Collagen Conc. (mg/ml)	Glacial Acetic Acid Conc. (% vol.)	Pore Morphology	Dry Max Pore Size (u)	Dry Avg Pore Size (u)	Max Particle Reduction after Wetting 9 Step (%)	2 Step (%)	Direct in PBS (%)	Direct in 70% ethanol (%)
1	spherical	Liquid N2		5	5	open	5 to 10	2.33	-1.6	0.6	67	41
2	deformed sphere[2]			3	5	open						
3	deformed sphere[2]			1	5	open						
4	spherical	Pentane	-15	5	5	open	20 to 30	3.41				
5	deformed sphere[2]		-15	3	5	open						
6	deformed sphere[2]		-15	1	5	open						
7	Non-Spherical	Air	-20	5	5	open	200	15.4		7.6	32.3	
7	Non-Spherical	Air	-20	5	5	open				-0.2 [1]	57.2 [1]	
7 replicate	Non-Spherical	Air	-20	5	5	open			6.0 [1]		48 [1]	
8	Non-Spherical	Air	-20	5	0.5	less open						
9	Non-Spherical	Air	-20	50	5	non-porous						
10	Non-Spherical	Air	-20	50	0.5	closed						
11	Disk Shaped Sheet						200					
12	Non-Spherical	Liquid N2		5	5	open	10 to 20	3.95				
13	Non-Spherical	Air	-80	5	5	open						

1. Reduction in cross-sectional area after wetting
2. Intent is to have spherical particle

The invention is further illustrated by the following examples, which should not be construed as further limiting.

A. SPHERICAL PARTICULATE SPONGES

Example 1 -*Effects of Formulation and Process Variables on Dry Sponge Porosity and Shape*

The intent here is to demonstrate the effects of collagen concentration, acetic acid concentration, collagen solubility, freezing temperature, and freezing medium on porosity and particle shape

Example 1B

Effects of Freezing Conditions and Collagen Conc. On Morphology

The effects of collagen concentration and cooling conditions are evaluated

Materials and Methods

Formulations listed in the table below were produced and imaged. For nos. 1 to 3 collagen spheres were prepared as follows. Insoluble, type I, bovine collagen from SIGMA was used for all samples. Collagen, acetic acid, and water were mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The resulting dispersion was added dropwise through a vibrating no. 22 needle into a bath of liquid nitrogen. Frozen specimens were lyophilized for 5 days at a pressure < 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days.

For samples 4 to 6 spheres are prepared as described above with the exception that the dispersion is added dropwise to a stirred pentane bath maintained at –15C to affect freezing. See casting apparatus in fig. 24.

For sample 7 specimens are prepared as described above with the exception that droplets of the dispersion are placed on to a silicone coated plastic film. The droplets spread out to form disk shaped structures. The whole assembly was placed, at room temperature, into a polystyrene foam insulated container. The container was then placed into a –20C freezer to affect slow cooling. Frozen specimens were lyophilized for 5 days at a pressure< 60 x 10^{-3}

MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days.

No	Collagen Concentration (mg/ml)	Glacial acetic acid (ml)	Deionized Water (ml)	Coolant	Coolant Temp (C)
1	5	10	190	Liquid N2	
2	3	10	190	Liquid N2	
3	1	10	190	Liquid N2	
4	5	10	190	Pentane	-15
5	3	10	190	Pentane	-15
6	1	10	190	Pentane	-15
7	5	10	190	air	-20
23	5	10	190	Pentane	-70

The specimens were imaged via SEM. Photomicrographs are shown in figs. 1 to 7.

Results and Conclusions:

Results are summarized as follows:

Cooling conditions have the biggest impact on pore size. The largest pores (>50u) are obtained for samples cooled at the slowest rate, slowly in air at –20C. See fig. 7. Intermediate pore size is obtained at the intermediate cooling rate, -15C in pentane. See figs 4 to 6. The smallest pores are obtained for the fastest cooling rate, liquid nitrogen. See figs 1 to 3. Small pores are also obtained for samples cast in pentane at –70C. These pores are similar in size to those for spheres cast in liquid nitrogen. See fig 23.

For samples made in liquid nitrogen, pore size is slightly affected by collagen concentration with the 3mg/ml and 1mg/ml having slightly larger pore size than that for the 5mg/ml.

For samples made in liquid pentane, pore size is similar at collagen concentrations of 5 and 3mg/ml. Pores are collapsed at a collagen concentration of 1mg/ml.

Collagen concentration has the biggest impact on particle shape. This comparison is only made for samples 1 to 6 since these are spherical after freezing. Sample 7 is disk shaped after freezing. Structures best approximating a sphere were obtained at a collagen concentration of 5mg/ml. See fig. 1A and fig. 4. Misshapen structures were obtained at a collagen concentration of 1 mg/ml. See fig 3 and fig. 6. At intermediate collagen concentration the particles are somewhat spherical.

Although this was not measured, frozen collagen spheres comprising the lowest collagen concentration shrank and disfigured the most in the lyophilization process than spheres comprising the higher collagen concentration. All samples were approximately spherical after freezing and prior to lyophilization. Additional shrinkage was not apparent during dehydrothermal cross-linking.

Example 1C

Effects of Freezing Conditions on Pore Size

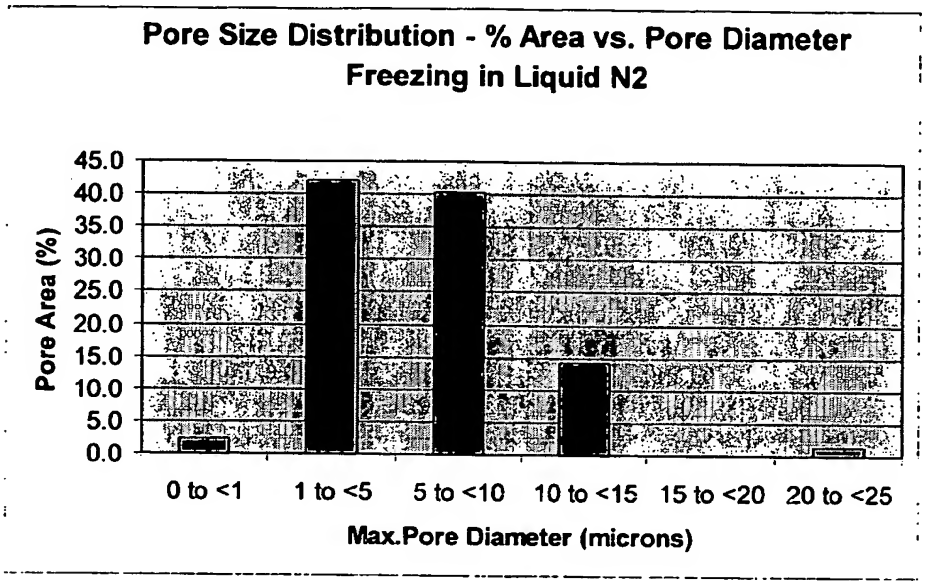
Materials and Methods:

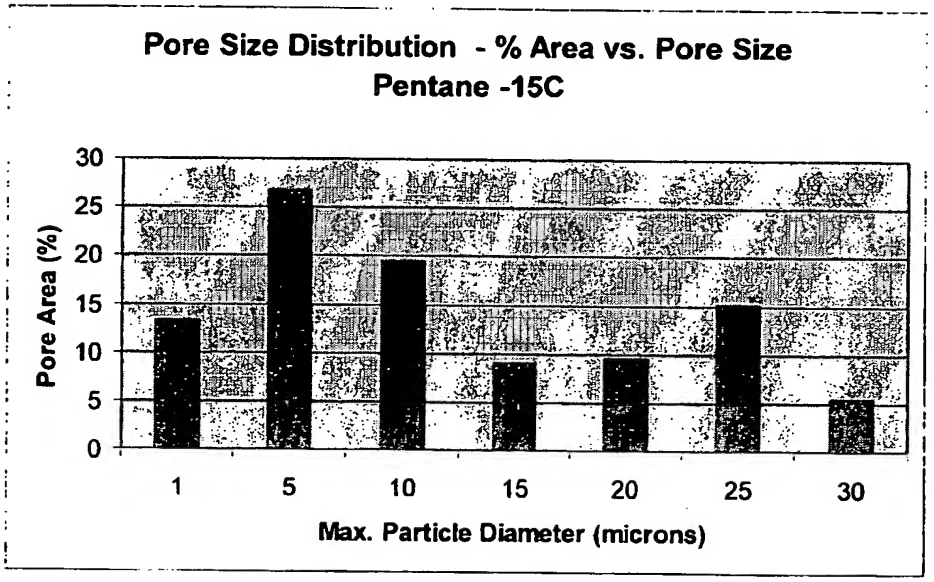
Pore size measurements were made for samples 1 and 4 from example 1B. Digital SEM photomicrographs were used for the measurements. These are shown in figs 25 to 29. There are 2 images for specimens made with liquid N2 cooling. The 2 specimens are from different batches. There are 3 images for specimens made with cooling in pentane at –15C. The 3 specimens are from 2 different batches.

Image Pro Plus 4.5 was used to analyze the digital SEM photomicrogtaphs. The protocol for the measurement process is as follows. From the main menu

-open image
-select measure
-select calibration
-select spatial
-set the spatial calibration
-close the calibration window
-select measure again from the main menu
-select count/size
-from the count/size window select measurements
-from the drop down box select diameter (max.) and area as the measurements to be made
-click ok
- from the count/size window make sure that automatic dark objects, measure objects, and apply filter ranges are all checked
-hit the count button
-to see data select view and then measurements from the count/size window

The area and max. particle diameter data are used to construct a plot of % of pore area as a function of pore diameter. Results are shown below. Avg. max. particle diameter is also calculated.





Results and Conclusions:

Results are summarized as follows

-For specimens frozen in liquid nitrogen., ~15% of the total area of the surface pores is occupied by pores >/= 10 microns in diameter.

-For specimens frozen in pentane at –15C.,~ 50% of the total area of the surface pores is occupied by pores >/= 10 microns in diameter

Example 1 E

Effects of Acid and Collagen Concentration and Freezing Conditions on Pore Size

Materials and Methods:

The intent is to evaluate the effects of freezing conditions, acid and collagen concentration on sponge pore size. Collagen dispersions were prepared with insoluble bovine collagen. Collagen, acetic acid, and water were mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The resulting dispersion was added dropwise through a vibrating no. 22 needle into a bath of pentane at –15C or liquid nitrogen. Frozen specimens were lyophilized for 5 days at a pressure< 60 MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days. Spheres that could be prepared were imaged via SEM. Magnification is in the range of 1000x to 2000x. This magnification range should be used for analyzing particles with mean max. pore diameter in the range of 2u to 4u (or max pore size of ~20u).

For a dispersion comprising 5mg/ml collagen and 5% glacial acetic acid two lots of spheres were cast in liquid nitrogen and 5 lots were cast in pentane at –15C. One lot each were cast in pentane at –15C for the following combinations

-5mg/ml collagen/3.5% glacial acetic acid

-3mg/ml collagen/2.5% glacial acetic acid

-5mg/ml collagen/2.5% glacial acetic acid

-5mg/ml collagen/0.5% glacial acetic acid

Multiple photos were imaged for each lot. Image Pro Plus 4.5 was used to analyze the digital SEM photomicrographs. The protocol for the measurement process is as follows. From the main menu

-open image
-select measure
-select calibration
-select spatial
-set the spatial calibration
-close the calibration window
-select measure again from the main menu
-select count/size
-from the count/size window select measurements
-from the drop down box select diameter (max.) and area as the measurements to be made
-click ok
- from the count/size window make sure that automatic dark objects, measure objects, and apply filter ranges are all checked
-hit the count button
-to see data select view from the count/size box and then select statistics to see the average values for max. diameter and area.

Results of pore size measurements are shown below. Average values for max. particle diameter and average area are shown.

No	Coolant	Temp C	collagen mg/ml	acid wt%	Mag	Mean Area Microns^2	Mean Max. Diam. microns
28-2	pentane	-15	5	3.5	1000	7.4	3.4
28-3	pentane	-15	5	3.5	2000	7.1	3.2
28-5	pentane	-15	5	3.5	1000	7.2	3.3
28-6	pentane	-15	5	3.5	2000	3.7	2.4
Avg.						6.35	3.075
15-2	Liq. N2		5	5	1700	2.22	1.97
15-4	Liq. N2		5	5	1700	2.76	2
4B	Liq. N2		5	5	1300	3.92	2.66
4IIA	Liq. N2		5	5	1300	5.3	2.7
Avg.						3.55	2.33
1B	pentane	-15	5	5	1300	14.8	4.18
1-IIB	pentane	-15	5	5	1300	15.7	3.83
1-111B	pentane	-15	5	5	1300	17.6	3.56
24-2	pentane	-15	5	5	1300	8.4	3.7
24-3	pentane	-15	5	5	1300	4.7	2.8
24-4	pentane	-15	5	5	1300	7.3	3.7
24-6	pentane	-15	5	5	1300	14.7	4.4
24-7	pentane	-15	5	5	1300	5.2	3.1
24-9	pentane	-15	5	5	1300	8.5	3.6
26-2	pentane	-15	5	5	1000	5.10	2.97
26-4	pentane	-15	5	5	2000	3.36	2.2
26-5	pentane	-15	5	5	1000	12.6	2.6
30-2	pentane	-15	5	5	1000	8.8	3.8
30-4	pentane	-15	5	5	1000	8.1	3.5
37-2	pentane	-15	5	5	1000	13.5	2.8
37-5	pentane	-15	5	5	1000	8.1	3.8
Avg.						9.84	3.41
29-2	pentane	-15	3	2.5	1000	4.9	3
29-3	pentane	-15	3	2.5	1000	4.9	2.9
29-5	pentane	-15	3	2.5	1000	4	2.8
29-6	pentane	-15	3	2.5	1000	3.5	2.5
Avg.							2.8
2	pentane	-15	5	2.5		can not be cast	
						can not be cast	
3	pentane	-15	5	0.5			

Results and Conclusions

-Formulations 2 and 3 could not be cast into spheres. Collagen particle size in the dispersion is too big to pump through no. 22 needle

-Spheres cast in pentane for formulation comprising 5 mg/ml collagen and 5% acid, exhibit the largest values for mean max. pore diameter and mean pore area. Pore size is significantly larger than that for the spheres cast in liquid nitrogen. Avg. (average of averages) max. diameters are 3.4u and 2.1u for the pentane and liquid nitrogen samples, respectively.

-For spheres cast in pentane at –15C lower acid or collagen does not result in larger pores

Example 1F

Spheres from Human Collagen

Collagen from human placenta Type VI from Sigma is used. A mixture of 5 mg/ml in 5% acetic acid are mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The resulting mixture is added dropwise through a vibrating no. 22 needle into a bath of liquid nitrogen. Frozen specimens are lyophilized for 5 days at a pressure < 60 x 10^{-3} MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days. The spheres are wetted by the stepwise wetting procedures described in other examples

Example 1G

Spheres from Recombinantly Produced Collagen

Recombinant Human Collagen I, 3 mg/ml in 10mM HCl, from Fibrogen is added dropwise through a vibrating no. 22 needle into a bath of liquid nitrogen. Frozen specimens are lyophilized for 5 days at a pressure < 60 x 10^{-3} MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days. The spheres are wetted by the stepwise wetting procedures described in the Exemplification.

Example 1H

Collagen Sponges Comprising Drug Added to Dispersion

A mixture of a water soluble or a water insoluble drug and 5 mg/ml of insoluble type I bovine collagen from SIGMA in 5% acetic acid is prepared. The mixture is mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture is metered with a peristaltic pump through a vibrating no. 22 needle dropwise into a bath of liquid nitrogen. Frozen specimens are lyophilized for 5 days at a pressure < 60 x 10-3 MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days. The spheres are wetted by the stepwise wetting procedures described in previous examples.

Example 1I

Collagen Sponges Wetted with Solution of Water Soluble Drug

Wetted sponges obtained in Example 14 are wetted with water, and transferred to a 0.2μ filter unit. The water is removed *via* filtration to a point where the wetted particulates are packed, but without a visible layer of liquid on top of the packed sponges. A solution of a water soluble drug is carefully added so that solution rests on top of layer of sponges. Drainage is allowed to occur until the liquid level just reaches the top of the layer of spheres.

Example 1J

Collagen Sponges Comprising Drug Added to Dispersion – Chemically Attached

A mixture of water soluble or water insoluble drug, a chemical agent to chemically bond the drug to collagen, and 5 mg/ml insoluble type I bovine collagen from SIGMA in 5% acetic acid is prepared. The mixture is mixed at 6000 rpm for 30 min at a temperature < 25C with a lab scale Silverson rotor / stator mixer. The mixture is metered with a peristaltic pump through a vibrating no. 22 needle dropwise into a bath of liquid nitrogen. Frozen specimens are lyophilized for 5 days at a pressure < 60 x 10-3 MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days. The spheres are wetted by the stepwise wetting procedures described in previous examples.

Example 2

Wet Sponge Morphology

The intent here is to demonstrate a gradient procedure for producing a wetted sponge with a morphology and pore size similar to that for its dry precursor. This is compared to a direct wetting procedure where morphology and pore size for the wetted sponge is significantly different from that for the dry precursor

Materials and Methods:

A formulation for a collagen dispersion is shown below

For 200ml of dispersion:	
Collagen Concentration	5 mg/ml
Acetic Acid Concentration	5%
Weight of Collagen	1g
Volume of Glacial acetic acid	10ml
Volume of DDW	190ml
Final volume of preparation	200ml

200 ml of the formulation is mixed for 30 min at 6000 rpm with a lab scale Silverson rotor stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion.

The dispersion was cast and frozen according to 3 different protocols as indicated in the table below.

No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
1	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	
2	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-15
3	droplet placed on release sheet	At room temp., place droplets on to release lines. Place assembly in insulate container. Place container in -20C freezer	air	-20

Frozen specimens are lyophilized for 5 days at < 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at < 1torr for 3 days

SEM photomicrographs were taken and are shown in Figs 1, 4, and 7

The sponges were wetted via 2 different procedures. One set of sponges was wetted directly into an aqueous nutrient solution. A second set was wetted according to the procedure shown below. The wetting in ethanol is done under reduced pressure for a short period of time to increase the wetting rate.

10. 100% ethanol
11. 85% ethanol / 15 % PBS (phosphate buffer solution)
12. 75% ethanol / 25% PBS
13. 65% ethanol / 35% PBS
14. 60% ethanol / 40% PBS
15. 50% ethanol / 50% PBS
16. 30% ethanol / 70% PBS
17. 15% ethanol / 85% PBS
18. 5% ethanol / 95% PBS

Three washings are then done with nutrient solution. Confocal images for the 6 different specimens are shown in figs. 9 to 14

Results and Conclusions:

A comparison is made between SEM images of the dry sponges and the confocal images of sponges.

-Compares figs 1 (dry SEM) to fig 9 (confocal/gradient wetting) and fig 10 (confocal/direct wetting).- Liquid N2 cooling

-Compares figs 4 (dry SEM) to fig 11 (confocal/gradient wetting) and fig 12 (confocal/direct wetting).- Liquid pentane @ -20C cooling

-Compares figs 7 (dry SEM) to fig 13 (confocal/gradient wetting) and fig 14 (confocal/direct wetting).- Air cooling @ -20C

Results are summarized as follows

-For samples wetted with a series of solvent/PBS mixtures the porosity of the sponge is similar to that for the dry spheres.

-Samples wetted directly in PBS solution have collapsed and pore size is very small in comparison to that for the dry sponges.

-Collapse and significant reduction of pore size occurs for sample wetted directly in PBS solution regardless of the pore size of the dry samples.

-Pore size reduction occurs even if the pores are large, as for samples made via the slow cooling process, when wetting is done directly into PBS. Compare fig 7 (dry sponge) with fig 14 (same formulation wetted directly into PBS)

Example 3

Sponge Dimensions

Materials and Methods

9 Step Gradient

Dry dehydrothermally cross-linked spheres were produced as for no. 1 in example 2. These were used for all of the measurements in example 3. The spheres were wetted by the same gradient wetting procedure as for example 2. Nine spheres were randomly selected for this experiment. The maximum diameter of the dry spheres were measured and the diameters were measured after the gradient wetting. These measurements were made manually with a stereo microscope . See table below. Note that average diameters are nearly identical.

	Dry Diameter dry	Diameter after Gradient Washing
	1.9	1.9
	1.8	2.3
	1.8	1.7
	1.8	1.8
	1.9	1.8
	1.7	1.6
	1.4	1.9
	2.1	1.9
	2	1.7
Average	1.81	1.84

2 Step Gradient

The diameters of 13 dry spheres were measured. The same spheres were wetted in 99.8% ethanol with the application of reduced pressure for a few minutes to facilitate wetting. Excess ethanol was removed from the spheres wetted with ethanol and the spheres were wetted in phosphate buffer solution. Incubate in phosphate buffer solution until all spheres sink to the bottom of the container. Diameters were measured. Note that average diameters are nearly identical.

	Dry diameter (mm)	Wet Diameter (mm)
	1	1.2
	2.5	1.2
	1.5	2.5
	1.5	2.5
	1.2	2
	2.5	2.5
	2.5	2
	1.2	1.5
	2.5	1.5
	1.5	1.2
	1.5	1
	1.2	1.2
	1	1.2
Average	1.66	1.65

Direct Wetting in Medium

The maximum diameters of 16 dry spheres were measured. The same 16 spheres were wetted directly in PBS. Reduced pressure was applied to facilitate wetting. See measurements below. Note the significant reduction in diameter after wetting directly in PBS

Dry Spheres Diam. mm	Spheres in PBS Diam. mm
1.2	0.5
1.2	0.4
1.2	0.4
1.2	0.6
2.2	0.5
2.6	0.5
2	0.4
1	0.5
1	0.6
1	0.5
2	0.4
2	0.5
1	0.5
1	0.4
1.4	0.5
1.4	0.5
Ave. 1.46	Ave. 0.48 ~67% shrink

Direct Wetting in 70% Ethanol

The maximum diameters of 14 dry spheres were measured. The same 14 spheres were wetted directly in 70% ethanol / 30% PBS. Reduced pressure was applied to facilitate wetting. See measurements below. Note the significant reduction in diameter after wetting directly in 70% ethanol / 30% PBS.

Dry Spheres Diam. mm	Spheres in 70% Ethanol Diam. mm
2	1
2.5	1
2	0.8
1.8	1.2
1.2	1
1	2
2	1
2	0.8
1.2	0.6
2	0.8
1.4	1
1.4	0.6
1.2	0.8
1.4	0.8
Ave. 1.65	Ave. 0.95
	~ 41% shrink

Results and Conclusions:

Results are summarized as follows

-The gradient wetting process results in virtually no shrinkage, even for the 2 step process comprising wetting in ethanol and then in medium

-Wetting directly in medium results in considerable shrinkage

-Wetting directly in 70% ethanol/ 30% PBS results in considerable shrinkage

Example 4A

Definition of Spherical and Measurements

Spherical is defined as follows. >/= 50% of particles in a population exhibit a roundness value of 1 to 1.2 using the equation

Roundness = (Perimeter ^{2}) / (4 * pi * area)

Dry Spheres

Dry spheres described as for no. 4 in example 1B were used for the measurements. Roundness is measured using a digital image of a population of spheres and Image Pro Plus 4.5. The protocol is identical to that described above except that roundness is chosen as the measurement to be made and from the count/size window make sure that automatic bright (not dark as for pore measurements) objects, measure objects, and apply filter ranges are all checked. A digital image of a population of the dry spheres is shown in fig 34. Roundness values are also shown. All values for this dry sphere population are between 1 and 1.2

Wet Spheres

Dry spheres described as for no. 4 in example 1B were used. These were subjected to the 9 step wetting procedure described in examples 2 and 3. Roundness is measured using a digital image of a population of spheres with Image Pro Plus 4.5 as described for dry spheres. A population of the wet spheres is shown in fig 35. The image was doctored prior to making measurements using the split object tool in Image Pro Plus 4.5. Roundness values are also shown. Note that 60% of spheres exhibit roundness values in the range of 1 to 1.2

B. NON-SPHERICAL PARTICULATE SPONGES

Example 5

Production of Non Spherical Particulate Collagen Sponges – Freezing in Air

Materials and Methods:

A formulation for a collagen dispersion is shown below

For 200ml of dispersion:	
Collagen Concentration	5 mg/ml
Acetic Acid Concentration	5%
Weight of Collagen	1gr
Volume of Glacial acetic acid	10ml
Volume of DDW	190ml
Final volume of preparation	200ml

The formulation is mixed for 30 min at 6000 rpm with a lab scale Silverson rotor stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The dispersion is poured into ice cube trays with dimensions of 1" x 1" x 1.75". Each tray is filled about 2/3 to 3/4 volume. The trays containing the dispersion are placed in a foam polystyrene container with a lid. The whole assembly is placed in a freezer set to –20C. The intent is to have slow cooling to generate a large pore size. The dispersion is chilled for, at least, 2 days at which point the dispersion is frozen.

Three frozen cubes are quickly removed from the cooler, split in half with a stainless steel knife, and added to a stainless steel dewer containing ~ 6 oz. of liquid nitrogen. The cubes in a liquid nitrogen medium are ground with a hand held high speed kitchen mixer. Grinding is done in 2 – 30 sec. periods.

The resulting dispersion of frozen particles in liquid nitrogen is poured into a series of sieves that are immersed in liquid nitrogen. See figure 33. The array is agitated to affect separation of particles according to size. Alternatively, the grinding and separation may be done in a single step, in liquid nitrogen, as shown in fig. 50. Frozen particle fractions were lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at < 1torr for 3 days.

The dehydrothermally cross-linked particles may be wetted in multistep processes as described in example 3 to preserve the porosity

SEM photomicrographs of the dry dehydrothermally cross-linked particulate sponges are shown in Figs. 37A. Confocal micrographs of the wetted particulate sponges are shown in Fig 37B.

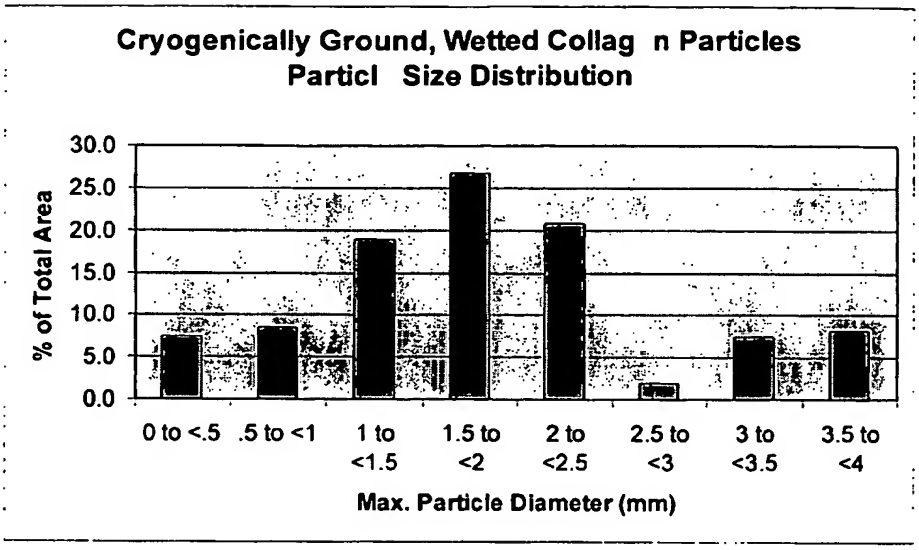
Example 5A

Characterization of Non Spherical Particulate Collagen Sponges –Particle Size –Method A

Particle size was measured for particles made as described in 5. Grinding and separation were done simultaneously. The fraction passing through a 1.5mm sieve and retained on a .5mm sieve was retained, lyophilized, and cross-linked. The particles were wetted in a stepwise process as described above. The dispersion of the wetted particles was agitated to break up aggregates of particles. The dry particles appear to be charged and form aggregates. An aqueous dispersion of the particles was imaged. Photomicrographs are shown in figs. 38.

The photomicrographs were analyzed using Image Pro Pius 4.5. Max. particle diameter, particle area, and particle roundness were measured. The protocol is identical to that described above in 4A except that area, max. particle diameter and roundness were measuredA particle size distribution is shown in fig below. The % of the total area is plotted vs. particle size. Note that some particles are larger than the pore size of the sieve that was used (1.5mm). There are 2 explanations. First particles may bond together during the cross-linking process. Second it is difficult to completely break up aggregates of particles.

The average roundness was 4.7



Example 5B

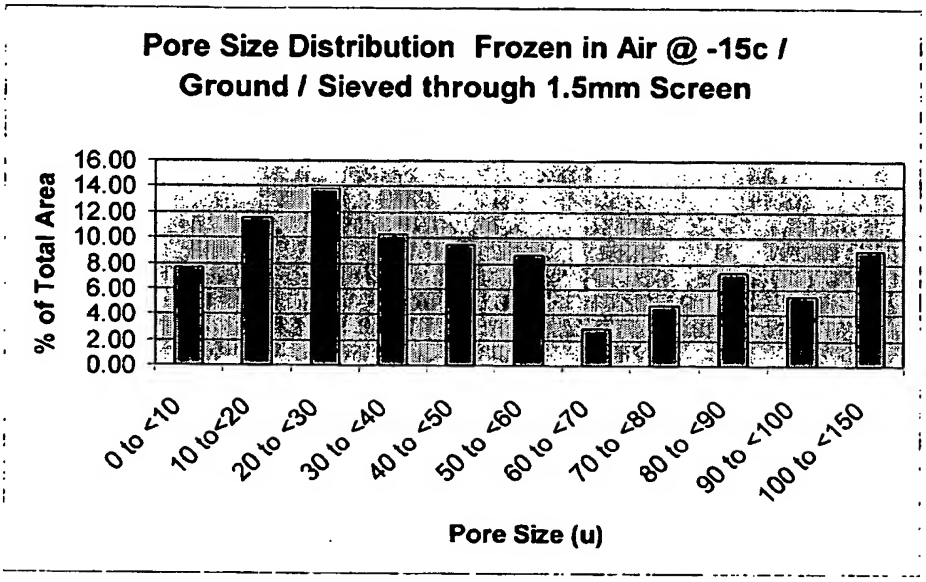
Characterization of Non Spherical Particulate Collagen Sponges -- Pore Size

Pore size was measured for the dry particles. The images shown in fig 41 were used. Two of the images are from a single batch. The other image is from a replicate batch. Image Pro Plus 4.5 was used to measure pore size. The protocol for the measurement process is as follows. From the main menu

-open image
-select measure
-select calibration
-select spatial
-set the spatial calibration
-close the calibration window
-select measure again from the main menu
-select count/size
-from the count/size window select measurements
-form the drop down box select diameter (max.) and area as the measurements to be made
-click ok
- from the count/size window make sure that automatic dark objects, measure objects, and apply filter ranges are all checked
-hit the count button

-to see data select view and then measurements from the count/size window

A plot is constructed of pore area as a function of max. particle diameter. See fig below.



Average max pore diameter and avg. pore area were also measured. For sponges with an average max. diameter in the range of 10u to 25u (max pore diameter of 100u) a magnification of 200x to 400x should be used for the digital images. Two photos from 2 separate batches were analyzed. Image Pro Plus 4.5 was used with the same protocol as described above in example 1E. Results are shown in the table below.

No	Coolant	Temp	Collagen	acid	Mag	Mean Area Microns^2	Mean Max. Diam. Microns
31-4	air	-20	5	5	200	219.3	19.1
31-6	air	-20	5	5	200	275	18.9
35-2	air	-20	5	5	350	93.4	10.6
35-5	air	-20	5	5	350	155	13.1
Avg.						185.7	15.4

Example 5C

Preparation and Characterization of Non Spherical Particulate Collagen Sponges – Particle Size – Method B

A formulation for a collagen dispersion is shown below

For 200ml of dispersion:

Collagen Concentration	5 mg/ml
Acetic Acid Concentration	5%
Weight of Collagen	1gr
Volume of Glacial acetic acid	10ml
Volume of DDW	190ml
Final volume of preparation	200ml

The formulation is mixed for 30 min at 6000 rpm with a lab scale Silverson rotor stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The dispersion is poured into ice cube trays with dimensions of 1" x 1" x 1.75". Each tray is filled about 2/3 to 3/4 volume. The trays containing the dispersion are placed in a foam polystyrene container with a lid. The whole assembly is placed in a freezer set to –20C. The intent is to have slow cooling to generate a large pore size. The dispersion is chilled for, at least, 2 days at which point the dispersion is frozen.

Three frozen cubes are quickly removed from the cooler, split in half with a stainless steel knife, and added to a 5mm stainless steel sieve suspended in a liquid nitrogen bath. The sieve is agitated with a shaker. The cubes, immersed in liquid, nitrogen are ground with a high speed kitchen type mixer. See fig 50. Ground particles, smaller than 5mm fall through the sieve. The ground frozen particles are separated, while immersed in liquid nitrogen, into 4 fractions

-3 to 5 mm
-2 to 3 mm
-.5 to 2 mm
-<0.5

Frozen particle fractions were lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days

The particle fractions were wetted in a stepwise process as described above. The dispersion of the wetted particles was agitated to break up aggregates of particles. The dry particles appear to be charged and form aggregates. Digital images of the wetted particles were recorded. Four images were recorded for each of the 2 largest particle fractions. The photomicrographs were analyzed using Image Pro Plus 4.5. Maximum avg. diameters were measured. The protocol is as follows

-From main menu open image
-From main menu select measure
-Select measurements
-From Features box select creating line
-Manually, measure max. particle diameter for each particle in image using this tool
-Select feature tab to see data

Raw data and averages are shown below for the 2 largest fractions

Maximum particle Diameter (mm)			
No.	3 to 5 mm sieve	No.	2 to 3 mm Sieve
1	2.27	1	3.85
2	2.73	2	5.36
3	4.82	3	6.98
4	3.67	4	2.08
5	7.27	5	4.36
6	19.82	6	1.60
7	2.31	7	2.19
8	3.27	8	3.05
9	3.59	9	1.20
10	4.83	10	5.22
11	3.76	11	2.22
12	6.23	12	3.70
13	5.01	13	1.00
14	3.22	14	1.53
15	2.76	15	2.20
16	3.90	16	2.26
17	2.34	17	1.50
18	1.58	18	1.70
19	5.22	19	3.53
20	4.44	20	1.98
21	3.74	21	2.07
22	4.21	22	4.98
23	3.77	23	1.93
24	4.54	24	1.77
25	3.80	25	4.80
26	2.68	26	1.95

27	7.50	27	2.61
28	2.44	28	2.74
29	3.25	29	3.37
30	8.49	30	9.98
31	2.20	31	6.77
32	5.29	32	3.42
Avg.	4.53	33	2.97
		34	0.77
		35	1.76
		36	2.12
		37	13.49
		38	5.71
		39	2.12
		40	1.92
		41	2.16
		42	4.40
		43	1.91
		44	2.24
		45	1.56
		46	2.07
		47	2.26
		48	1.69
		49	3.64
		50	1.40
		51	2.22
		52	4.76
		53	2.25
		54	3.88
		55	7.70
		56	2.80
		57	2.02
		Avg.	3.22

Example 6

Production of Non Spherical Particulate Collagen Sponges – Freezing in Liquid Nitrogen

Materials and Methods:

A formulation for a collagen dispersion is shown below

For 200ml of dispersion:	
Collagen Concentration	5 mg/ml
Acetic Acid Concentration	5%
Weight of Collagen	1gr
Volume of Glacial acetic acid	10ml
Volume of DDW	190ml
Final volume of preparation	200ml

The formulation is mixed for 30 min at 6000 rpm with a lab scale Silverson rotor stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. With a 25 ml pipette large droplets (> 10mm) of the dispersion are dropped into liquid nitrogen and allowed to freeze. The large droplets are added to a 3mm stainless steel sieve suspended in a liquid nitrogen bath. The sieve is agitated with a shaker. The droplets, immersed in liquid, nitrogen are ground with a high speed kitchen type mixer. See fig 50. Ground particles, smaller than 3mm fall through the sieve. The ground frozen particles are separated, while immersed in liquid nitrogen, into 3 fractions

-2 to 3 mm
-.5 to 2 mm
-< .5 mm

Frozen particle fractions were lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days.

Example 6B

Characterization of Non Spherical Particulate Collagen Sponges – Pore Size

Pore size was measured for the dry particles described in example 6. Average max pore diameter and avg. pore area were measured. For sponges with a max pore diameter of 10 - 20u a magnification of 1000x should be used for the pore size analysis. The images shown in fig 53 were used. The images are for the 2 largest fractions described in example 6. Image Pro Plus was used to measure pore size. The protocol for the measurement process is as follows. From the main menu

-open image
-select measure
-select calibration
-select spatial
-set the spatial calibration
-close the calibration window
-select measure again from the main menu
-select count/size
-from the count/size window select measurements
-form the drop down box select diameter (max.) and area as the measurements to be made
-click ok
- from the count/size window make sure that automatic dark objects, measure objects, and apply filter ranges are all checked
-hit the count button
-to see data select view from the count/size window and then statistics to see average values
Results are shown below

No	Coolant	Temp	Collagen Mg/ml	Sieve Size	acid %	Mag	Mean Area Microns ^{2}	Mean Max. Diam. microns
51-2	Liq. N2		5	2 to 3	5	1000	12.6	4.1
51-3	Liq. N2		5	2 to 3	5	1000	9.6	3.8
Avg.							11.1	3.95
52-2	Liq. N2		5	.5 to 2	5	1000	9.8	3.7
52-3	Liq. N2		5	.5 to 2	5	1000	10.3	3.8
Avg.							10.05	3.75

Example 7

Wet vs. Dry SPONGE Dimensions

Materials and Methods:

A formulation for a collagen dispersion is shown below

For 200ml of dispersion:	
Collagen Concentration	5 mg/ml
Acetic Acid Concentration	5%
Weight of Collagen	1gr
Volume of Glacial acetic acid	10ml
Volume of DDW	190ml
Final volume of preparation	200ml

The formulation is mixed for 30 min at 6000 rpm with a lab scale Silverson rotor stator mixer. The mixture is stored overnight in a cooler above the freezing point of the dispersion. The dispersion is poured into ice cube trays with dimensions of 1" x 1" x 1.75". Each tray is filled about 2/3 to 3/4 volume. The trays containing the dispersion are placed in a foam polystyrene container with a lid. The whole assembly is placed in a freezer set to –20C. The intent is to have slow cooling to generate a large pore size. The dispersion is chilled for, at least, 2 days at which point the dispersion is frozen.

Three frozen cubes are quickly removed from the cooler, split in half with a stainless steel knife, and added to a 5mm stainless steel sieve suspended in a liquid nitrogen bath. The sieve is agitated with a shaker. The cubes, immersed in liquid, nitrogen are ground with a high speed kitchen type mixer. See fig 50. Ground particles, smaller than 5mm fall through the sieve. The ground frozen particles are separated, while immersed in liquid nitrogen, into 3 fractions

-3 to 5 mm
-2 to 3 mm
-< 2mm

Frozen particle fractions were lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at < 1torr for 3 days.

Two sets of samples from each of the 2 largest fractions were imaged. Maximum particle diameter and particle area were measured with Image Pro Plus 4.5. One set from each of the 2 largest fractions was wetted in a 2 step procedure

-in absolute ethanol

One set from each of the 2 largest fractions was wetted directly in medium. The 4 set of samples were imaged. Maximum particle diameter and particle area were measured with Image Pro Plus 4.5.

	Millimeters ^{2}	Millimeters
	Avg. Area	Avg. Max. D
Dry Particles - 3 to 5 mm sieve	4.35	3.03
Same as above wetted directly in medium	1.86	2.05
% Reduction	57.2	32.3
Dry Particles - 3 to 5 mm sieve	4.9	3.27
Same as above wetted in 2 step process	4.91	3.02
% Reduction	-0.2	7.6
Dry Particles - 2 to 3 mm sieve	2.43	2.31
Same as above wetted in 2 step process	2	2.01
% Reduction	17.7	13.0
Dry Particles - 2 to 3 mm sieve	2.53	2.28
Same as above wetted directly in medium	1.29	1.75
% Reduction	49.0	23.2

Results and Conclusions:

Results are summarized as follows

-Shrinkage is excessive for particles wetted directly into medium. Avg. particle cross-sectional area is reduced by about 50%

-There is no shrinkage or minimal shrinkage for particles wetted in the 2 step process. Values range from ~0 to 17%

Example 8

Cells cultured on Particulate sponges

Materials and Methods

Spheres made in Pentane at −15C

Spheres from no. 4 of example 1B were used. They were wetted via the 9 step process described above. They were further washed 3 times with medium prior to being seeded with porcine fibroblasts. About 200 ml of collagen microspheres, stored in D-MEM at 4 C, were transferred to a 500-ml filter apparatus with a 0.2 micron filter. The culture medium was removed by suction and 200 ml of F12/D-MEM medium containing 15% of fetal calf serum, 2 mM glutamine, 1x penicillin/streptomycin, 0.39 mg/ml of L-arginine, 0.19 mg/ml sodium pyruvate, 2 µg/ ml of putrescine, 8 µg/ ml of insulin and 8 µg/ ml of hydrocortisone were added to the drained microspheres. The microspheres were transferred to a sterile 500 ml bottle using a 25 ml pipette.

For study, 9 ml of the washed micropheres were pipetted into a sterile 6-well plate insert, with a diameter of 2.4 cm and a 74 microns mesh at the bottom, in a sterile culture dish with a 10 cm diameter. The cultured medium in each insert was allowed to drain by gravity. Then, the drained microspheres were washed with 10 ml of F12/DMEM and the medium again was drained by gravity. The washing process was repeated one more time. Then, the drained microspheres were transferred to another sterile 6-well plate insert with a diameter of 2.4 cm and a 0.4 micron mesh at the bottom of the insert in a 10 cm diameter sterile culture dish, using a sterile spatula. The insert was then placed in a 100 mm sterile petri dish. About 20 ml of the full F12/DMEM medium were added to the dish but not into the insert. Three million fibroblasts in 1 ml of full F12/DMEM medium were added into the insert with the washed and drained microspheres. The dish was then incubated at 37 C in a CO_2 incubator for 2 to 3 hr to facilitate the adsorption of the cells onto the microspheres. After the incubation, more medium was added to the dish until the medium covered the opening of the insert in the dish. The total volume in the dish was about 50 to 60 ml of culture medium. The dish was then incubated at 37 C in a CO_2 incubator for 4 to 6 days. At the time indicated, the microspheres with the cells were pipetted into another 74 micron insert to drain all the culture medium. The microspheres were then washed with 1x phosphate buffered saline in a 6-well plate before they were fixed with 10% formalin for 2 hrs. The microspheres were then washed extensively in the insert. Then, they were stained and analyzed by confocal microscopy.

Confocal photomicrographs in fig. 39

Spheres made in Liquid Nitrogen

Spheres from no. 1 of example 1B were used. They were wetted via the 9 step process described above. They were further washed 3 times with medium prior to be seeded with porcine fibroblasts. These were seeded with cells and cultured in vitro as described above in protocol for spheres made in pentane at –15C

Confocal photomicrographs are shown in fig. 42

Particle frozen in air at –20C

Particles from no. 7 of example 1B were used. They were wetted via the 9 step process described above. They were further washed 3 times with medium prior to be seeded with porcine fibroblasts. These were seeded with cells and cultured in vitro as described above in protocol for spheres made in pentane at –15C

Confocal photomicrographs are shown in fig. 51

Example 9

Apparatus for Simultaneous Grinding and Sorting

An apparatus for simultaneous grinding and sorting is shown if fig 50. Large particles of frozen dispersion are added to the sieze. A high speed mixer is used to reduce particle size. The ground particles are expelled from the sieve as they are reduced to a particle size less than the sieve openings. The vortex created by the grinder facilitates this removal. Agitation of the sieve also promotes removal of the ground particles.

This process permits production of particles with a narrow range of particle sizes in comparison to that produced in a process where grinding and separation are done separately.

Example 10

Collagen/Chondroitin 6 Sulphate Composites

Materials and Methods:

A collagen dispersion comprising 5 mg/ml collagen and 5% glacial acetic acid was prepared as described above. A solution of 5 mg/ml sodium salt of chondroitin 6 sulphate was also prepared. For the 1st preparation 4 parts of the collagen solution and 1 part of the C6S solution were mixed on a shaker for 15 min. Precipitation occurred. The mixture was poured into ice cube trays. The trays containing the dispersion are placed in a foam polystyrene container with a lid. The whole assembly is placed in a freezer set to –15C. The intent is to have slow cooling to generate a large pore size. The dispersion is chilled for, at least, 2 days at which point the dispersion is frozen.

Three frozen cubes are quickly removed from the cooler, split in half with a stainless steel knife, and added to a basket constructed of a 3 mm stainless steel sieve. The basket is immersed in liquid nitrogen. While the basket is agitated, the cubes are ground with a high speed mixer. The fractured particles pass through the 3mm sieve. The resulting particles are then filtered through a .5mm sieve. The particles that remain on the sieve were lyophilized for 5 days at a pressure< 60×10^{-3} MBAR. The lyophilized sponges were dehydrothermally cross-linked at 120C at <1torr for 3 days.

It should be noted that dehydrothermally cross-linked, collagen sponges (*e.g.*, wetted and dry particulates, *e.g.*, non-spherical) of the invention may comprise a glycosamine glycan. In certain embodiments, as in this example, the glycosamine glycan is chondroitin 6 sulphate.

Results and Conclusions:

An SEM photomicrograph of particles is shown in Fig 52, frame 44-1. Pore size was measured with Image Pro Plus 4.5 as described above.

No	Coolant	Temp C	Collagen Mg/ml	Size	Acid %	Mag x	Mean Area Microns^2	Mean Max. Diam. microns
44-2	air	-20	4/1 (C/C6S) pre		5	200	867	22
45-1	air	-20	4/1 (C/C6S) tray		5	200	578	17.8
45-3	air	-20	4/1 (C/C6S) tray		5	60	7098	63

Example 11

Small Particles by High Intensity Mixing

Small, particulate collagen sponges, wetted in an aqueous medium are prepared as follows.

(1) A biopolymer dispersion or a biopolymer solution is prepared.

(2) The solution or dispersion is metered into a water insoluble organic solvent while mixing with a high intensity mixer and maintaining the temperature below the freezing point of the biopolymer solution or biopolymer dispersion to produce a 2 phase mixture comprising a disperse phase and a continuous phase. The continuous phase is the water insoluble organic solvent and the disperse or discreet phase comprises a frozen biopolymer solution or a frozen biopolymer dispersion. To accomplish this the biopolymer solution or dispersion is added to the agitated organic solvent in such a way so as to impart very high shear from the moment that the aqueous composition enters the organic solvent bath. In one embodiment, a rotor stator mixer is used to create the high shear.

The intent is to produce small particles. To accomplish this, the biopolymer solution or dispersion must be added to the agitated organic solvent in such a way so as to impart very high shear from the moment that the aqueous composition enters the organic solvent bath. One option is to use a rotor stator mixer and meter the aqueous solution or dispersion as shown in the images shown below (Image 1 and Image 2).

Image1
Rotor/Stator High Shear Mixer

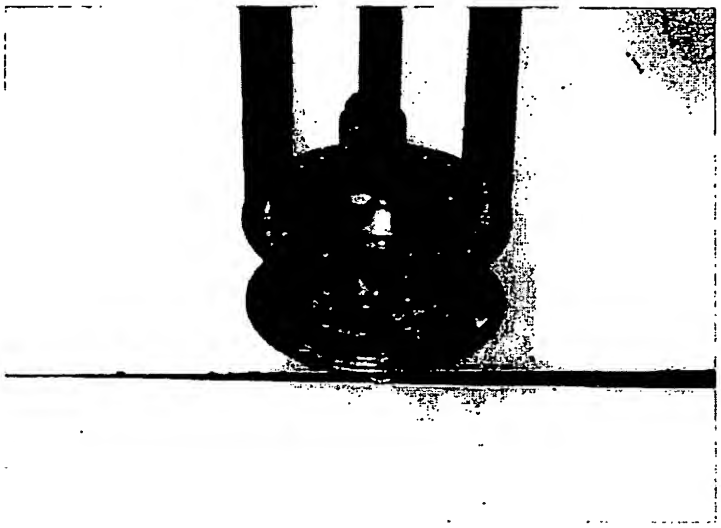
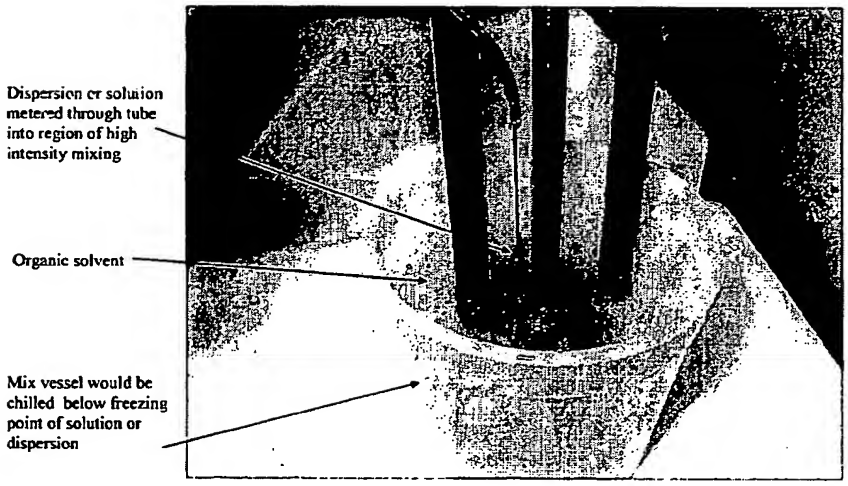


Image 2



(3) The frozen particles are separated from the organic solvent while maintaining the temperature below the freezing point of the biopolymer solution or biopolymer dispersion.

(4) The frozen particles are lyophilized to produce dry porous particles.

(5) The dry porous particles are cross-linked.

(6) The cross-linked sponges is wetted in a non-aqueous water soluble solvent at reduced pressure, resulting in cross-linked sponges wetted with a non-aqueous medium.

(7) The wetted, dehydrothermally cross-linked sponges are exposed to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium. (Alternatively the sponges are directly washed in an aqueous medium.)

Example 12

Small Particles by Spraying into a Chilling Bath

Small particulate collagen sponges may be prepared by one of the three following methods.

1. Small particulate collagen sponges are prepared by
   - atomizing a dispersion of insoluble collagen or a solution of soluble collagen into a cryogenic bath by metering the dispersion or solution of collagen through a nozzle that is immersed in the cryogenic bath.
   - Lyophilizing the frozen particles
2. Small particulate collagen sponges are prepared by
   - atomizing a dispersion of insoluble collagen or a solution of soluble collagen into a cryogenic bath by metering the dispersion or solution of collagen through a nozzle that is immersed in the cryogenic bath.
   - Lyophilizing the frozen particles
   - Cross-linking
3. Small particulate collagen sponges wetted in an aqueous medium are prepared by
   - atomizing a dispersion of insoluble collagen or a solution of soluble collagen into a cryogenic bath by metering the dispersion or solution of collagen through a nozzle that is immersed in the cryogenic bath.
   - Lyophilizing the frozen particles
   - Cross-linking
   - wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium
   - exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium. Alternatively wash directly in an aqueous medium

Example 13

Fast Multiple Step Wetting Process

Dry collagen particles or spheres were added to a container comprising ethanol (absolute) and then transferred to a bell shape vacuum desiccator. Vacuum was then applied for 5 min to release all the air bubbles trapped in the pores, and the collagen particles sank to the bottom of the container. The ethanol wetted collagen particles or spheres were transferred to a filter unit (0.2micron).

The ethanol is then removed by filtration to a point where the wetted particulates are packed without a visible layer of ethanol on top of the packed particles. 50% ethanol/50% phosphate buffer solution (PBS) was added to the filter unit (70%EtOH/30%PBS does not work because a fine white precipitate forms in the solution). The particles or spheres were allowed to equilibrate with the ethanol/PBS mixture for about 10 min. The ethanol/PBS mixture was removed *via* filtration to a point where the wetted particulates were packed, but without a visible layer of ethanol/PBS mixture, on top of the packed spheres.

The processes of washing and filtering was then repeated with 100% PBS and then with 1 x DMEM. After removing the 1 x DMEM by suction, 2 x the volume of the packed volume of the particles or spheres of 1 x DMEM containing 10% fetal calf serum and penicilin and streptomycin were added. The suspension was stirred and allowed to equilibrate for 10 min. The suspension was then transferred to a sterile bottle and stored at 4 C for at least one to two days. Before use, the microspheres suspension was transferred into a filter apparatus (0.2 micron) and washed once as described previously with 1 X DMEM containing 10% fetal calf serum and penicillin and streptomycin.

After removing the medium by filtration, 2 x volume of the packed particles or spheres of the same culture medium were added. The particles or spheres suspension is transferred to a sterile bottle and was ready to be used. The wetted particles or spheres were kept at 4C.

Alternatively, after washing the particles or spheres with 1 x DMEM, the washing process can be repeated twice with 1 x DMEM containing 10% fetal calf serum and penicilin and streptomycin. After the final wash, 2 x the volume of the packed volume of the particles or spheres of 1 x DMEM containing 10% fetal calf serum and penicillin and streptomycin are added. The particles or spheres suspension is then transferred to a sterile bottle and is ready to be used. Again, the wetted particles or spheres are kept at 4C.

Example 14

Fast 2 Step Wetting Process

Dry collagen particles or spheres were added to a container comprising ethanol (absolute) and then transferred to a bell shape vacuum desiccator. Vacuum was then applied for 5 min to release all the air bubbles trapped in the pores, and the collagen particles sank to the bottom of the container. The ethanol wetted collagen particles or spheres were transferred to a filter unit (0.2micron).

The ethanol is then removed by filtration to a point where the wetted particulates are packed without a visible layer of ethanol on top of the packed particles. Water or PBS (phosphate buffer solution) was added to the filter unit. The particles or spheres were allowed to equilibrate for about 10 min. The water or PBS was then removed *via* filtration to a point where the wetted particulates are packed but without a visible layer of liquid on top of the packed spheres. The processes of washing and filtering is repeated with water or PBS. The spheres may then be wetted with DMEM as described in 13.

Example 15

Compositions and Processes Comprising Hydroxy Apatite

A mixture comprising 1 mg/ml to 10mg/ml of collagen and hydroxyapatite in 1% to 10% glacial acetic acid is prepared, wherein the minimum percentage of collagen in the collagen + hydroxy apatite mixture is 5%. The mixture is poured into ice cube trays. The trays containing the dispersion are then placed in a foam polystyrene container with a lid. The whole assembly is placed in a freezer set to –15C. The intent is to have slow cooling to generate a large pore size. The dispersion is chilled for, at least, 2 days at which point the dispersion is frozen.

Three frozen cubes are quickly removed from the cooler, split in half with a stainless steel knife, and added to a basket constructed of a 3 mm stainless steel sieve. The basket is immersed in liquid nitrogen. While the basket is agitated, the cubes are ground with a high speed mixer. The fractured particles pass through the 3mm sieve. The resulting particles are then filtered through a 0.5mm sieve. The particles that remain on the sieve are lyophilized for 5 days at a pressure< 60 x 10^{-3} MBAR. The lyophilized sponges are dehydrothermally cross-linked at 120C at <1torr for 3 days.

The dry particles are wetted by stepwise wetting procedures already described for other particulates.

Compositions incorporating hydroxy apatite, a significant component of the

extrcellular matrix in bone (collagen being another major component of the extracellular matrix in bone), are useful alone, or in composites, as implantable bone tissue supplements.

Example 16

Islets – Microspheres Comprising Cells with Shell of Complex Coacervate

Spheres made in Pentane at –15C

Spheres from Sample no. 4 of example 1B are wetted *via* the 9 step process described above. They are further washed 3 times with medium, prior to being seeded with porcine fibroblasts. About 200 ml of collagen microspheres, stored in D-MEM at 4 C, are transferred to a 500-ml filter apparatus with a 0.2 micron filter. The culture medium is removed by suction and 200 ml of F12/D-MEM medium containing 15% of fetal calf serum, 2 mM glutamine, 1x penicillin/streptomycin, 0.39 mg/ml of L-arginine, 0.19 mg/ml sodium pyruvate, 2 µg/ ml of putrescine, 8 µg/ ml of insulin and 8 µg/ ml of hydrocortisone are added to the drained microspheres. The microspheres are transferred to a sterile 500 ml bottle using a 25 ml pipette.

For study, 9 ml of the washed microspheres are pipetted into a sterile 6-well plate insert, with a diameter of 2.4 cm and a 74 microns mesh at the bottom, in a sterile culture dish with a 10 cm diameter. The cultured medium in each insert is allowed to drain by gravity. Then, the drained microspheres are washed with 10 ml of F12/DMEM and the medium again was drained by gravity. The washing process is repeated one more time. Then, the drained microspheres are transferred to another sterile 6-well plate insert with a diameter of 2.4 cm and a 0.4 micron mesh at the bottom of the insert in a 10 cm diameter sterile culture dish, using a sterile spatula. The insert is then placed in a 100 mm sterile Petri dish. About 20 ml of the full F12/DMEM medium are added to the dish but not into the insert. Three million fibroblasts in 1 ml of full F12/DMEM medium are added into the insert with the washed and drained microspheres. The dish is then incubated at 37 C in a CO_2 incubator for 2 to 3 hr to facilitate the adsorption of the cells onto the microspheres. After the incubation, more medium is added to the dish until the medium covered the opening of the insert in the dish. The total volume in the dish is about 50 to 60 ml of culture medium. The dish is then incubated at 37 C in a CO_2 incubator for 4 to 6 days.

The calcium level is adjusted and the microspheres comprising cells are incubated. The microspheres comprising cells are added to an alginate solution. Upon addition a complex coacervate shell forms around the microspheres comprising cells

Example 17A

Continuous Process for the Manufacture of Dermal Membrane

(a) Preparation of particulate collagen dispersion

About 200 mL of dehydrothermally cross-linked dry collagen particulates prepared according to the methods of the invention were suspended in 200 mL of absolute ethanol, in a sterile 500-mL conical flask with a screw cap. The suspension was subjected to a vacuum to remove air in the particulates.

After the particulates sank to the bottom of the flask, the liquid was removed by first decanting, followed by using a pipette. About 200 mL of 70% ethanol in PBS was added to the flask, which was then shaken with a wrist shaker to mix the suspension until all the particulates sank to the bottom of the flask. The liquid was subsequently removed as previously described.

About 200 mL of 50% ethanol in PBS were then added, the suspension was shaken, and the liquid was removed after the particulates sank to the bottom of the flask. The process was repeated, continuing with 30% ethanol in PBS, 100% PBS. A photomicrograph of the particulate dispersion is shown below

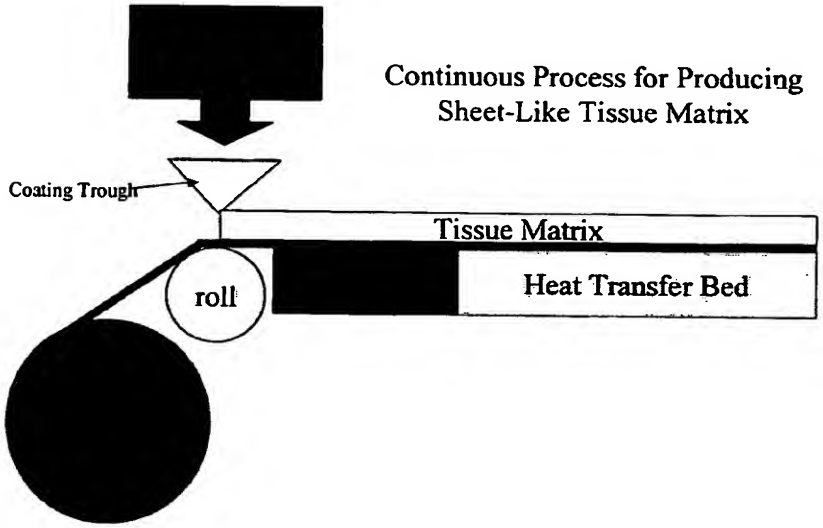
Finally D-MEM containing 10% fetal calf serum supplemented with glutamine and penicillin/streptomycin. The particulates were stored in D-MEM at 4 °C.

(b) Preparation of cell dispersion in a gellable collagen solution

Acid soluble collagen solution (0.35 mL), containing 1 x D-MEM and 10% fetal calf serum at 4 °C, was mixed with D-MEM (0.2 mL) containing 10% fetal calf serum and (1 X 10^5) normal human fibroblasts at 4 °C.

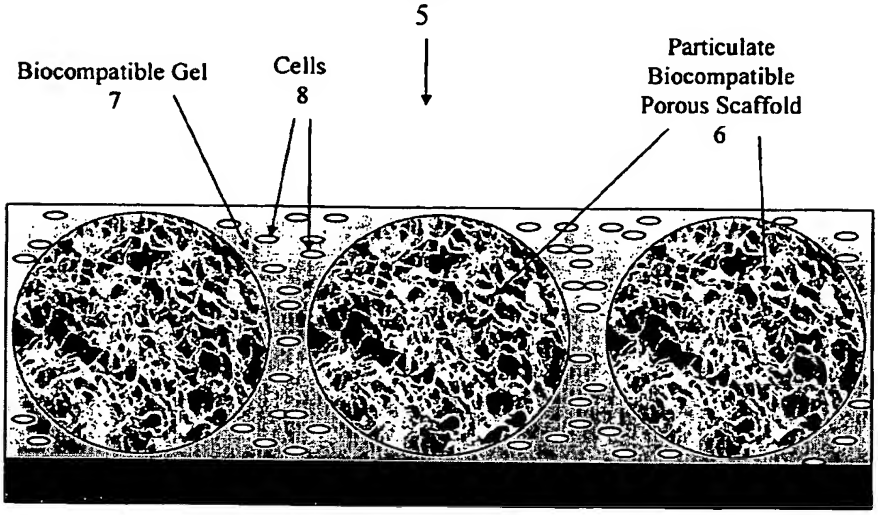
(c) Preparation of tissue composite using collagen particulates

The particulate collagen dispersion of (a) and the cell dispersion of (b) are mixed in a ratio of 1.5/0.45 to 3/0.45 while maintained at a temperature of 4C. The mixture is added to the coater of the apparatus shown below. The mixture is coated on to the moving polymer film. Excess culture medium is removed *via* suction through the porous film by the suction bed as shown below while still maintaining the temperature at ~ 4C. The coated film is then heated to ~ 37C by the heat transfer bed and gellation of the collagen solution occurs.



A schematic of the tissue matrix is shown in the figure below

Particulate Biopolymer Scaffold Dispersed in a Biopolymer Gel Seeded with Cells



The sheet-like composite may be cut into the shape desired for use. It is stored in culture medium until application

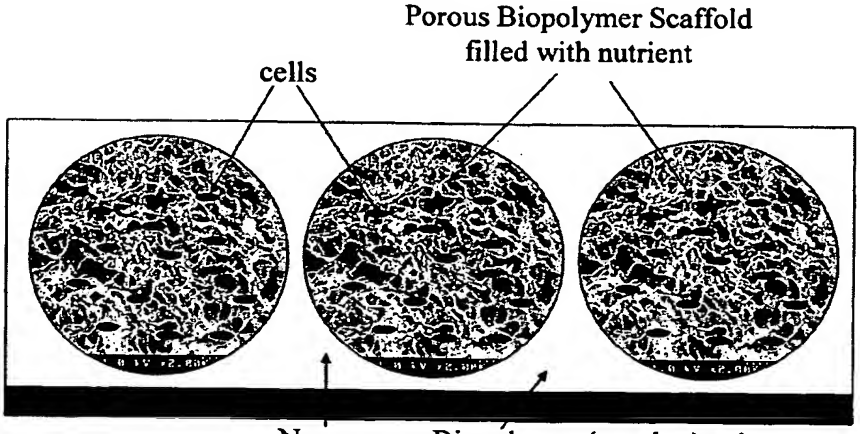
Example 17B

Continuous Process for the Manufacture of Dermal Membrane

A non-spherical particulate collagen particle is prepared in accordance with the processes of the invention. An aqueous dispersion of the particles is prepared as described in Example 17A part (a) above. The particle dispersion is mixed with a cell dispersion. The volume of cell culture medium is maintained at a level just greater than that required to wet the ingredients. The mixture is maintained in a quiescent state to allow the cells to attach. Additional medium is added and cells are culture in a bioreactor to the desired density.

The dispersion particulate collagen with attached cells is mixed with a gellable collagen solution and the temperature maintained at ~ 4C. The mixture is added to the hopper of the apparatus shown in Example 17A part (c). An engineered tissue composite is produced in a similar manner as that described in Example 17A part (c). A schematic of the composite is shown in the figure below

Dispersion of Particulate, Biopolymer Scaffold Particles,
Filled with Nutrient Solution, in a Biopolymer Gel
Cells Seeded in Scaffold

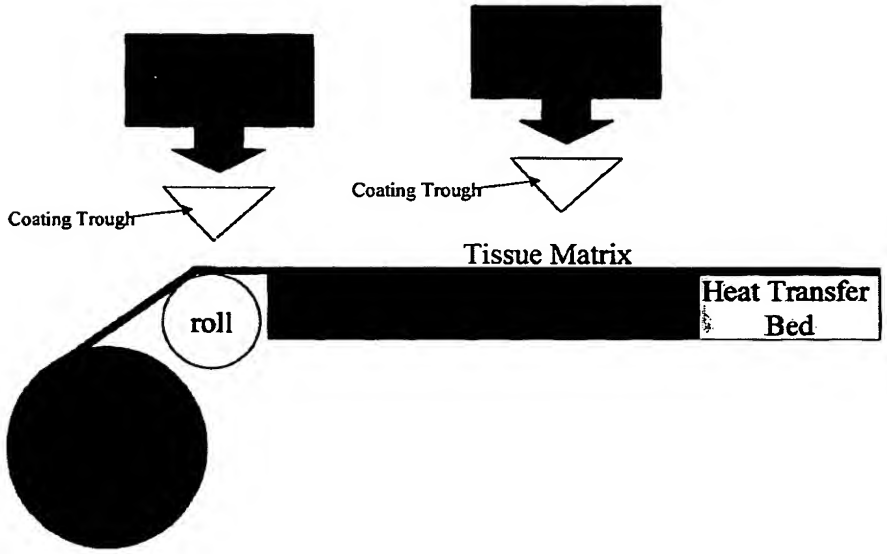


Example 17C

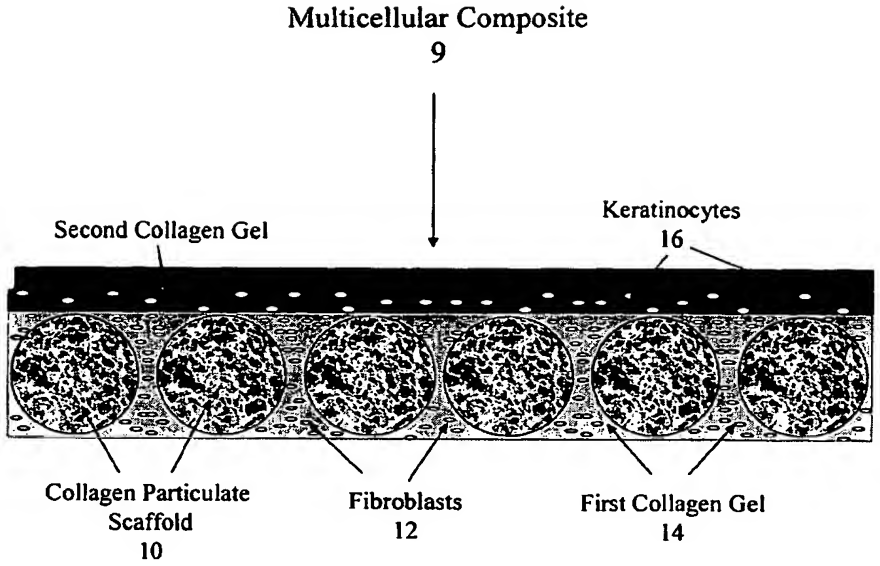
Continuous Process for the Manufacture of Dermal Membrane

This is an example of a process to produce a 2 layer tissue matrix (the apparatus is shown below. There are 2 coating stations. The 2nd coating station is used to coat a dispersion of cells in a gellable collagen solution to the composite produced in example 17A to form the composite shown below.

Continuous Process for Producing
Sheet-Like Tissue Matrix



A dispersion of cells in a gellable collagen solution is coated at the 1st coating station and temperature is maintained below the gelling temperature. A dispersion of particulate collagen and cells in a gellable collagen solution is coated at the 2nd coating station while the temperature is maintained below the gel temperature. Optionally, excess nutrient medium is removed through the porous film via the suction bed. The bi-layer tissue matrix heated to the gel temperature on the heat transfer bed to gel the composite. A schematic of the finished product is shown below


Multicellular Composite
9
Second Collagen Gel
Keratinocytes
16
Collagen Particulate
Scaffold
10
Fibroblasts
12
First Collagen Gel
14

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

INCORPORATION BY REFERENCE

The entire contents of all patents, published patent applications, and referenced figures (*e.g.*, Figures 1A-1C, 1C-2nd Batch, 2, 2A-2B, 3, 3A-3B, 4, 4A-4E, 4- 2nd Batch, 4A-4B – 2nd Batch, 5, 5A-5B, 6, 6A, 7, 9, 10, 11, 12, 13, 14, 23, 23A, 23B, 24, 25, 26, 27, 28, 29, 33, 34, 35, 37A-37B, 38 41, 39, 42, 51, 50, 52, 53, 54, 54 (cont-1), 54 (cont-2), 55, 56, 57 and 58)f and other references cited herein are hereby expressly incorporated herein in their entireties by reference.

CLAIMS

Composition Claims/Composition by Method

1. A dehydrothermally, cross-linked collagen sponge wetted with an aqueous medium wherein the volume of the wetted sponge is within ±20% of the value for volume of the unwetted sponge.

1a. The collagen sponge of claim 1, wherein the volume of the wetted sponge is within ±10% of the value for volume of the unwetted sponge.

1b. The collagen sponge of claim 1, wherein the volume of the wetted sponge is within ±5% of the value for volume of the unwetted sponge.

2. A dehydrothermally, cross-linked collagen sheet sponge wetted with an aqueous medium wherein the cross-sectional area or volume of the wetted sponge is within ±20% of the value for cross-sectional area or volume of the unwetted sponge.

2a. The collagen sponge of claim 1, wherein the volume of the wetted sponge is within ±10% of the value for cross-sectional area or volume of the unwetted sponge.

2b. The collagen sponge of claim 1, wherein the volume of the wetted sponge is within ±5% of the value for cross-sectional area or volume of the unwetted sponge.

3. A spherical, dehydrothermally cross-linked, collagen sponge wherein the average maximum diameter of the pores on the surface of the particle is 2.5μ to 5μ.

3aa. A spherical, dehydrothermally cross-linked, collagen sponge wherein the average maximum diameter of the pores on the surface of the particle is 3μ to 5μ.

3ab. A spherical, dehydrothermally cross-linked collagen sponge wherein the average area of the pores on the surface of the particle is > 4 mm^2.

3a. The collagen sponge of 3, wherein diameter is 0.25 to 10 mm.

3b. The collagen sponge of 3, wherein diameter is 0.5 to 3 mm.

4. A spherical, dehydrothermally cross-linked, collagen sponge wherein >/= 30% of the surface pore area is occupied by pores that have a maximum diameter of >/= 10 microns.

5. A population of spherical, dehydrothermally cross-linked, collagen sponges wetted with an aqueous medium, wherein spherical is defined as having >/= 50% of the population exhibit a roundness value between 1 and 1.25, wherein roundness is defined as

$$\text{Roundness} = (\text{Perimeter}^2) / (4*\text{pi}*\text{area}).$$

6. A population of spherical, dehydrothermally cross-linked, collagen sponges wetted with an aqueous medium wherein the maximum average diameter or the average cross-sectional area or the average volume of wetted sponges are within ±20% of the value for the maximum average diameter or the average cross-sectional area or the average volume of the unwetted sponge.

6a. The population of collagen sponges of claim 6, wherein the maximum average diameter or the average cross-sectional area or the average volume of the wetted sponges is within ±10% of the value for the maximum average diameter or the average cross-sectional area or the average volume of the unwetted sponge.

6b. The population of collagen sponges of claim 6, wherein the maximum average diameter or the average cross-sectional area or the average volume of the wetted sponges is within ±5% of the value for the maximum average diameter or the average cross-sectional area or the average volume of the unwetted sponge.

7. A population of spherical, dehydrothermally cross-linked, collagen sponges wherein >/= 30% of the surface pore area is occupied by pores that have a maximum diameter of >/= 10 microns and spherical is defined as having >/= 50% of the population exhibit a roundness value between 1 and 1.2, wherein roundness is defined as

$$\text{Roundness} = (\text{Perimeter}^2) / (4*\text{pi}*\text{area}).$$

8. A particulate, man-made, non-spherical, dehydrothermally cross-linked, collagen sponge.

9. A particulate, man-made, non-spherical, dehydrothermally cross-linked, wetted collagen sponge.

9a. A population of non-spherical, dehydrothermally cross-linked, collagen sponges wetted with an aqueous medium wherein the average cross-sectional area or volume or max. diameter of wetted sponges are ± 20% of the value for the average cross-sectional area or volume or max. diameter for the unwetted sponge.

9b. The population of collagen sponges of claim 9a, wherein the average cross-sectional area or volume or max. diameter of the wetted sponges is within ±10% of the value for the average cross-sectional area or volume or max. diameter for the unwetted sponge.

9c. The population of collagen sponges of claim 9a, wherein the maximum average diameter or the average cross-sectional area or volume or max. diameter of the wetted sponges is within ±5% of the value for the average cross-sectional area or volume or max. diameter for the unwetted sponge.

10. A population of particulate sponges of claim 8, 9, and 9a, wherein the average roundness is >/= 2.

11. The collagen sponges of claim 8, wherein >/= 50% of the total cross-sectional area of population of sponges is made up by particles with a diameter ranging from 1 to 2.5 mm.

12. The collagen sponges of claim 8, wherein the average max. pore diameter is 3μ to 16μ.

12a. The collagen sponges of claim 8 wherein the average pore area is 10 to 200mm^2.

13. The preferred collagen sponges of claim 9 wherein the average max. particle diameter is 0.5 to 10 mm.

14. The collagen sponges of claim 9 wherein the average max. particle diameter is 0.1 to 25mm.

15. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(a) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(b) casting the dispersion or the solution into a shape desired for end use;

(c) freezing the cast shape;

(d) lyophilizing the frozen, cast shape to form a collagen sponge;

(e) dehydrothermally cross-linking the lyophilized collagen sponge;

(f) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(g) exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium.

15a. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(a) preparing a dehydrothermally cross-linked collagen;

(b) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(c) exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium.

16b. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(a) preparing a dehydrothermally crosslinked collagen;

(b) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(c) washing or wetting with an aqueous medium.

16. A dehydrothermally cross-linked, collagen sponge (particulate sponges and non-particulate sponges) wetted with an aqueous medium prepared by a method comprising:

(a) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(b) casting the dispersion or the solution into a shape;

(c) freezing the cast shape;

(d) milling the shape into particles at a temperature below the freezing point of the particles;

(e) lyophilizing the frozen particles to form collagen sponges;

(f) dehydrothermally cross-linking the lyophilized collagen sponges;

(g) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(h) exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an

aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium.

17. A particulate, dehydrothermally cross-linked, collagen sponge prepared by a method comprising:

(i) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(j) casting the dispersion or the solution into a shape;

(k) freezing the cast shape;

(l) milling the shape into particles at a temperature below the freezing point of the particles in a coolant medium;

(m) separating the milled particles into ranges by sieving in the coolant medium;

(n) lyophilizing the frozen particles to form collagen sponges; and

(o) dehydrothermally cross-linking the lyophilized collagen sponges.

18. A particulate, dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(p) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(q) casting the dispersion or the solution into a shape;

(r) freezing the cast shape;

(s) milling the shape into particles at a temperature below the freezing point of the particles in a coolant medium;

(t) separating the milled particles into ranges by sieving in the coolant medium;

(u) lyophilizing the frozen particles to form collagen sponges;

(v) dehydrothermally cross-linking the lyophilized collagen sponges;

(w) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(x) exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an

aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium.

19. A particulate, dehydrothermally cross-linked, collagen sponge prepared by a method comprising:

(y) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(z) casting the dispersion or the solution into a shape;

(aa) freezing the cast shape;

(bb) simultaneously milling and sieving the shape into particles at a temperature below the freezing point of the particles in a coolant medium;

(cc) lyophilizing the frozen particles to form collagen sponges; and

(dd) dehydrothermally cross-linking the lyophilized collagen sponges.

20. A particulate, dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(ee) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(ff) casting the dispersion or the solution into a shape;

(gg) freezing the cast shape;

(hh) simultaneously milling and sieving the shape into particles at a temperature below the freezing point of the particles in a coolant medium lyophilizing the frozen particles to form collagen sponges;

(ii) dehydrothermally cross-linking the lyophilized collagen sponges;

(jj) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent at reduced pressure, resulting in dehydrothermally cross-linked sponges wetted with a non-aqueous medium; and

(kk) exposing the wetted, dehydrothermally cross-linked sponges to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water or an aqueous solution to form a dehydrothermally cross-linked sponges wetted with an aqueous medium.

21a. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(a) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(b) casting the dispersion or the solution into a shape desired for end use;

(c) freezing the cast shape;

(d) lyophilizing the frozen, cast shape to form a collagen sponge;

(e) dehydrothermally cross-linking the lyophilized collagen sponge;

(f) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

(g) washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21aa. The method of claim 21a, wherein step (g) involves washing with a series of non-aqueous water soluble solvent / water mixtures starting with a mixture comprising a high level of the non-aqueous water soluble solvent and then stepwise with mixtures comprising progressively higher levels of water.

21ab. The method of claim 21a, wherein the lyophilized collagen sponge is subjected to milling in a cryogenic medium prior to dehydrothermally cross-linking the lyophilized collagen sponge.

21ac. The method of claim 21ab, wherein the milled particles are separated into ranges by sieving in a cryogenic medium after milling and before lyophilization.

21ae. The method of claim 21a, wherein the lyophilized collagen sponge is subjected to simultaneously milling and sieving the shape into particles in a cryogenic medium prior to dehydrothermally cross-linking the lyophilized collagen sponge.

21af. The method of claim 21a, wherein step (b) involves pumping the dispersion or solution through a narrow tube into air.

21ag. The method of claim 21a, wherein step (b) involves casting a shape in a mold.

21ah. The method of claim 21a, wherein the freezing medium of (c) is air, a gas, liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent.

21aaa. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(h) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(i) spraying the collagen solution or a collagen dispersion through a nozzle directly into a chilling bath that may include liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent;

(j) lyophilizing the frozen, cast shape to form a collagen sponge;

(k) dehydrothermally cross-linking the lyophilized collagen sponge;

(l) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

(m)washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21aab. The method of claim 21ag, wherein the collagen sponge wetted with an aqueous medium are less than or equal to 100μ in diameter.

21aac. A dehydrothermally cross-linked, collagen sponge wetted with an aqueous medium prepared by a method comprising:

(n) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(o) adding a collagen solution or a collagen dispersion to a rapidly stirred chilling bath that may include liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent.;

(p) lyophilizing the frozen, cast shape to form a collagen sponge;

(q) dehydrothermally cross-linking the lyophilized collagen sponge;

(r) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

(s) washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21aad. The method of claim 21bc, wherein the collagen sponge wetted with an aqueous medium are less than or equal to 100μ in diameter.

21b. A biopolymer sponge wetted with an aqueous medium prepared by a method comprising:

(a) preparing an aqueous dispersion of insoluble biopolymer or solution of soluble biopolymer;

(b) casting the dispersion or the solution into a shape desired for end use;

(c) freezing the cast shape;

(d) lyophilizing the frozen, cast shape to form a biopolymer sponge;

(e) cross-linking the biopolymer sponge;

(f) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

(g) washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21ba. The method of claim 21b, wherein step (g) involves washing with a series of non-aqueous water soluble solvent / water mixtures starting with a mixture comprising a high level of the non-aqueous water soluble solvent and then stepwise with mixtures comprising progressively higher levels of water.

21bb. The method of claim 21b, wherein the lyophilized collagen sponge is subjected to milling in a cryogenic medium prior to dehydrothermally cross-linking the lyophilized collagen sponge.

21bc. The method of claim 21bb, wherein the milled particles are separated into ranges by sieving in a cryogenic medium after milling and before lyophilization.

21bd. The method of claim 21b, wherein the lyophilized collagen sponge is subjected to milling in a cryogenic medium prior to dehydrothermally cross-linking the lyophilized collagen sponge.

21be. The method of claim 21b, wherein the lyophilized collagen sponge is subjected to simultaneously milling and sieving the shape into particles in a cryogenic medium prior to dehydrothermally cross-linking the lyophilized collagen sponge.

21bf. The method of claim 21b, wherein step (b) involves pumping the dispersion or solution through a narrow tube into air.

21bg. The method of claim 21b, wherein step (b) involves casting a shape in a mold.

21bh. The method of claim 21b, wherein the freezing medium of (c) is air, a gas, liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent.

21bj. The method of claim 21b, wherein the biopolymer may include a protein, a carbohydrate, a peptide, a lipid, or a combination thereof.

21bk. A biopolymer sponge wetted with an aqueous medium prepared by a method comprising:

(t) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(u) spraying the collagen solution or a collagen dispersion through a nozzle directly into a chilling bath that may include liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent;

(v) lyophilizing the frozen, cast shape to form a collagen sponge;

(w) dehydrothermally cross-linking the lyophilized collagen sponge;

(x) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

(y) washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21bl. The method of claim 21bk, wherein the collagen sponge wetted with an aqueous medium are less than or equal to 100μ in diameter.

21bm. A biopolymer sponge wetted with an aqueous medium prepared by a method comprising:

(z) preparing an aqueous dispersion of insoluble collagen or solution of soluble collagen;

(aa) adding a collagen solution or a collagen dispersion to a rapidly stirred chilling bath that may include liquid nitrogen, a cryogenic liquid, or a water-insoluble organic solvent.;

(bb) lyophilizing the frozen, cast shape to form a collagen sponge;

(cc) dehydrothermally cross-linking the lyophilized collagen sponge;

(dd) wetting the dehydrothermally cross-linked sponges in a non-aqueous water soluble solvent; and

washing the sponges wetted with a non-aqueous water soluble solvent with an aqueous solution.

21bn. The method of claim 21bm, wherein the collagen sponge wetted with an aqueous medium are less than or equal to 100μ in diameter.

21. The sponges of the current inventions wherein collagen sources include animal, human, and synthetic

22. The sponges of the current inventions wherein collagen types include type I to XXI including I, II, III, and IV.

23. The sponges of the current invention, wherein optional ingredients added to the collagen dispersion or collagen solution prior to casting and freezing include proteins, carbohydrates, and lipids.

24. The sponges of the current invention, wherein a 0.05% to 10.0% dispersion of insoluble or soluble collagen is used

25. The sponges of the current invention, wherein a 0.1% to 1.0% dispersion of insoluble or soluble collagen is used

26. The sponges of the current invention, wherein a 0.3% to 0.7% dispersion of insoluble or soluble collagen is used

27. The sponges of the current invention, wherein the collagen dispersion comprises 1% to 20% glacial acetic acid

28. The sponges of the current invention, wherein the collagen dispersion comprises 1% to 5% glacial acetic acid

29. The sponges of the current inventions wherein dehydrothermal cross-linking is performed at a temperature between 80C and 150C

30. The sponges of the current inventions wherein dehydrothermal cross-linking is performed at a pressure of less than 5 torr.

31. The sponges of the current inventions wherein dehydrothermal cross-linking is performed at a pressure of less than 1 torr

32. The wetted sponges, wherein the non-aqueous solvent is ethanol, isopropanol, methanol, acetone, dimethyl ether, other water soluble alcohols and ketones.

33. A process for wetting sponges with a sequence of five wetting agents and the sequence of five wetting agents comprises:

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 65% non-aqueous, water soluble solvent/6% to 35% water;

64% to 35% non-aqueous, water soluble solvent/ 36% to 65% water;

34% to 6% non-aqueous, water soluble solvent/ 66% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95% water.

34. A process for wetting sponges with a sequence of four wetting agents and the sequence of four wetting agents comprises

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 50% non-aqueous, water soluble solvent/6% to 50% water;

49% to 6% non-aqueous, water soluble solvent/ 51% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95%.

35. A process for wetting sponges with a sequence of two wetting agents and the sequence of two wetting agents comprises:

100% to 95% non-aqueous, soluble solvent; and water.

36. Particulate, non-spherical, dehydrothermally cross-linked, collagen sponges comprising a glycosamine glycan.

37. Particulate, non-spherical, dehydrothermally cross-linked, wetted collagen sponges comprising a glycosamine glycan.

38. The particulates of 36 and 37 wherein the glycosamine glycan is chondroitin 6 sulphate.

39. A porous, particulate biopolymer sponge wetted with an aqueous medium with a particle size < 100μ, wherein the cross-sectional area or volume of the wetted sponge is within +/- 20% of the value of the cross-sectional area or volume for the unwetted sponge.

40. The biopolymer sponge of claim 39, wherein the cross-sectional area or volume of the wetted sponge is within +/- 10% of the value of the cross-sectional area or volume for the unwetted sponge.

41. The biopolymer sponge of claim 39, wherein the cross-sectional area or volume of the wetted sponge is within +/- 5% of the value of the cross-sectional area or volume for the unwetted sponge.

41a. The biopolymer sponge of claim 39, wherein the biopolymer is collagen.

Applications

42. A carrier device comprising a wetted spherical and/or non-spherical particulates, of the present invention, and a microorganism.

42a. The carrier device of claim 42, wherein the microorganism is cells or bacteria.

43. A carrier device comprising the wetted spherical and/or non-spherical particulates, of the present invention, and cells.

44. An aqueous dispersion or slurry comprising the spherical and/or non-spherical particulates, of the present invention

45. An aqueous dispersion or slurry comprising the spherical and/or non-spherical particulates, of the present invention, and a microorganism.

46. A medical sealant comprising an aqueous dispersion or slurry comprising the spherical and/or non-spherical particulates, of the present invention.

47. An enclosure comprising the wetted spherical and/or non-spherical particulates, of the present invention.

48. An enclosure comprising the wetted spherical and/or non-spherical particulates, of the present invention and a microorganism.

49. An enclosure comprising the wetted spherical and/or non-spherical particulates, of the present invention and cells.

50. The enclosures of 41 to 43 and 47 to 49 wherein at least one face of the enclosure is living tissue.

51. A molded or cast object comprising a wetted spherical and/or non-spherical particulates, of the present invention

52. The enclosures of 41 to 43 and 47 to 49 wherein one face of the enclosure is a porous fabric.

53. The composites described above cultured in vivo.

54. The composites described above cultured in vitro.

55. A composite comprising the spherical and/or non-spherical particulates and a pharmaceutical agent.

56. A cell-based drug delivery device comprising wetted collagen particles of the present invention.

57. The drug delivery device of 56, wherein the cell is modified to express the desired drug.

58. A drug delivery device comprising the wetted collagen particle of the present invention and a small molecule and or a large molecule drug.

59. The device of 58 wherein the drug is incorporated prior to dehydrothermal cross-linking of the particulate.

60. The device of 58 wherein the drug is incorporated after dehydrothermal cross-linking of the particulate.

61. Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and an antibiotic.

62. Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a growth factor.

63. Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a steroid.

64. Another embodiment of the invention is a drug delivery device comprising wetted collagen particles of the present invention and a spermicidal agent.

65. The compositions and processes of the present invention wherein the aqueous medium is sterile water, a nutrient solution supportive of cell growth, a phosphate buffer solution, or a buffer solution.

66. The composites of 42 and 43 that are coated with a complex coacervate.

67. A process for preparing the composites of 66 wherein the composites of 42 or 43 also comprise one component of a complex coacervate and these compositions are added to a solution comprising the 2nd component of the complex coacervate.

68. Sponges of the present invention to be used as a support for culturing cells in a bioreactor.

69. A continuous process for preparing sheet-like single layer and multiple layer engineered tissue matrices comprising cells, a particulate biopolymer scaffold, and a biopolymer gel.

70. The process of claim 69 further comprising the following steps:

(a) mixing an aqueous dispersion of a particulate biopolymer scaffold with cells dispersed in a solution of a gellable biopolymer at a temperature at which the gellable biopolymer solution will not gel;

(b) casting the mixture of cells, particulate biopolymer scaffold, and biopolymer gel on to a film in a continuous web process; and

(c) heating the mixture to a temperature at which the gellable biopolymer solution gels.

71. The process of claim 69 further comprising the following steps:

(a) cells are cultured on a particulate biopolymer scaffold in an aqueous medium that supports cell growth to produce an aqueous dispersion of cells attached to the particulate biopolymer scaffolds;

(b) an aqueous dispersion of cells attached to the particulate biopolymer scaffolds is mixed with a solution of a gellable biopolymer at a temperature at which the gellable biopolymer solution will not gel;

(c) the mixture of cells attached to particulate biopolymer scaffolds, and biopolymer gel are cast on to a film in a continuous web process; and

(d) the mixture is heated to a temperature at which the gellable biopolymer solution gels.

72. The process of claim 69 further comprising the following steps:

(a) preparing a dispersion of a particulate biopolymer scaffold and cells in a solution of a gellable biopolymer at a temperature at which the gellable biopolymer solution will not gel;

(b) casting the mixture of cells, particulate biopolymer scaffold, and biopolymer gel on to a film in a continuous web process; and

(c) heating the mixture to a temperature at which the gellable biopolymer solution gels.

73. A processes for producing multiple layer matrices comprising

a first layer prepared by the process of claims 70, 71, or 72; and

a second layer prepared by the process of claims 70, 71, or 72; or comprising cells dispersed in a biopolymer gel; or comprising an aqueous dispersion of cells wherein the second layer is cast on to the first layer in a continuous web process.

74. The processes of claims 70-73, wherein the polymer film is porous and excess aqueous medium is removed from the mixture of cells, biopolymer scaffold, and gellable biopolymer solution prior to gellation of the gellable biopolymer solution.

75. The processes of claims 70-74, wherein the particulate biopolymer scaffold comprises collagen.

76. The processes of claims 70-74, wherein the gellable biopolymer solution is a collagen solution.

77. A composite as describe in Example 17B, with or without the porous film.

78. The composite of claim 77, wherein the dry collagen sponges are wetted with a sequence of five wetting agents, and the sequence of five wetting agents comprises:

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 65% non-aqueous, water soluble solvent/6% to 35% water;

64% to 35% non-aqueous, water soluble solvent/ 36% to 65% water;

34% to 6% non-aqueous, water soluble solvent/ 66% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95% water.

79. The composite of claim 77, wherein the dry collagen sponges are wetted with a sequence of four wetting agents and the sequence of four wetting agents comprises:

100% to 95% non-aqueous, water soluble solvent/ 0% to 5% water;

94% to 50% non-aqueous, water soluble solvent/6% to 50% water;

49% to 6% non-aqueous, water soluble solvent/ 51% to 94% water; and

0% to 5% non-aqueous, water soluble solvent/ 100% to 95%.

80. The composite of claim 77, wherein the dry collagen sponges are wetted with a sequence of two wetting agents and the sequence of two wetting agents comprises:

100% to 95% non-aqueous, soluble solvent; and water.

81. A filter media comprising spherical and/or non-spherical particulates, of the present invention.

82. A chromatography media comprising the wetted spherical and/or non-spherical particulates of the present invention.

83. A device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids.

84. The device of claim 83, wherein the polymer comprises a naturally occurring biopolymer.

85. The device of claim 84, wherein the biopolymer is a protein, polysaccharide, or lipid.

86. The device of claim 83, wherein the polymer is water-swellable.

87. The device of claim 83, wherein the interior of the container is contact with the interpenetrating network.

88. The device of claim 83, wherein the volume fraction of biopolymer is 0.1 to 50%.

89. The device of claim, 83 wherein the diameter of the continuous voids is 0.1 microns to 200 microns.

90. The device of claim 83, wherein the device is used for chromatographic separations of molecules.

91. The device of claim 84, wherein the biopolymer is cross-linked.

92. The device of claim 91, wherein the biopolymer is dehydrothermally cross-linked.

93. The device of claim 91, wherein the biopolymer is chemically cross-linked.

94. The device of claim 91, wherein the biopolymer is cross-linked by radiation.

95. The device of claim 83, wherein the continuous polymer network comprises a surfactant.

96. The device of claim 84, wherein the biopolymer is collagen.

97. A method of producing device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids comprising the following steps:

producing an aqueous solution and/or a dispersion of a polymer;

filling a tube with the solution and/or dispersion of a polymer;

freezing the solution and/or dispersion of a polymer in the container; and

lyophilizing the container filled with the frozen solution and or dispersion of a polymer.

98. The method of claim 97, wherein the aqueous solution or dispersion further comprises an organic solvent.

99. The method of claim 97, wherein the aqueous solution or dispersion is frozen in a bath maintained at a temperature below the freezing point of the solution and or dispersion of the polymer.

100. The method of claim 99, wherein the bath is liquid nitrogen.

101. A method of producing a device comprising a container enclosing a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids comprising the following steps:

producing an aqueous solution and/or a dispersion of a polymer;

freezing the solution and or dispersion of a polymer in the shape of the container;

lyophilizing the shaped, frozen solution and/or dispersion of a polymer to form a monolithic interpenetrating network comprising a continuous polymer network and a continuous network of voids into a container.

inserting the shaped, lyophilized, monolithic interpenetrating network into the container

sealing the monolith into the container to insure that the monolith is in contact with interior wall of the container.

102. A method of claim 101 wherein contact between the monolith and the container wall is established by hydrating the lyophilized monolith inside the tube.

103. A method of claims 97 and 101, wherein the lyophilized monolith is further subjected to the steps of:

wetting in a non-aqueous water soluble solvent and then

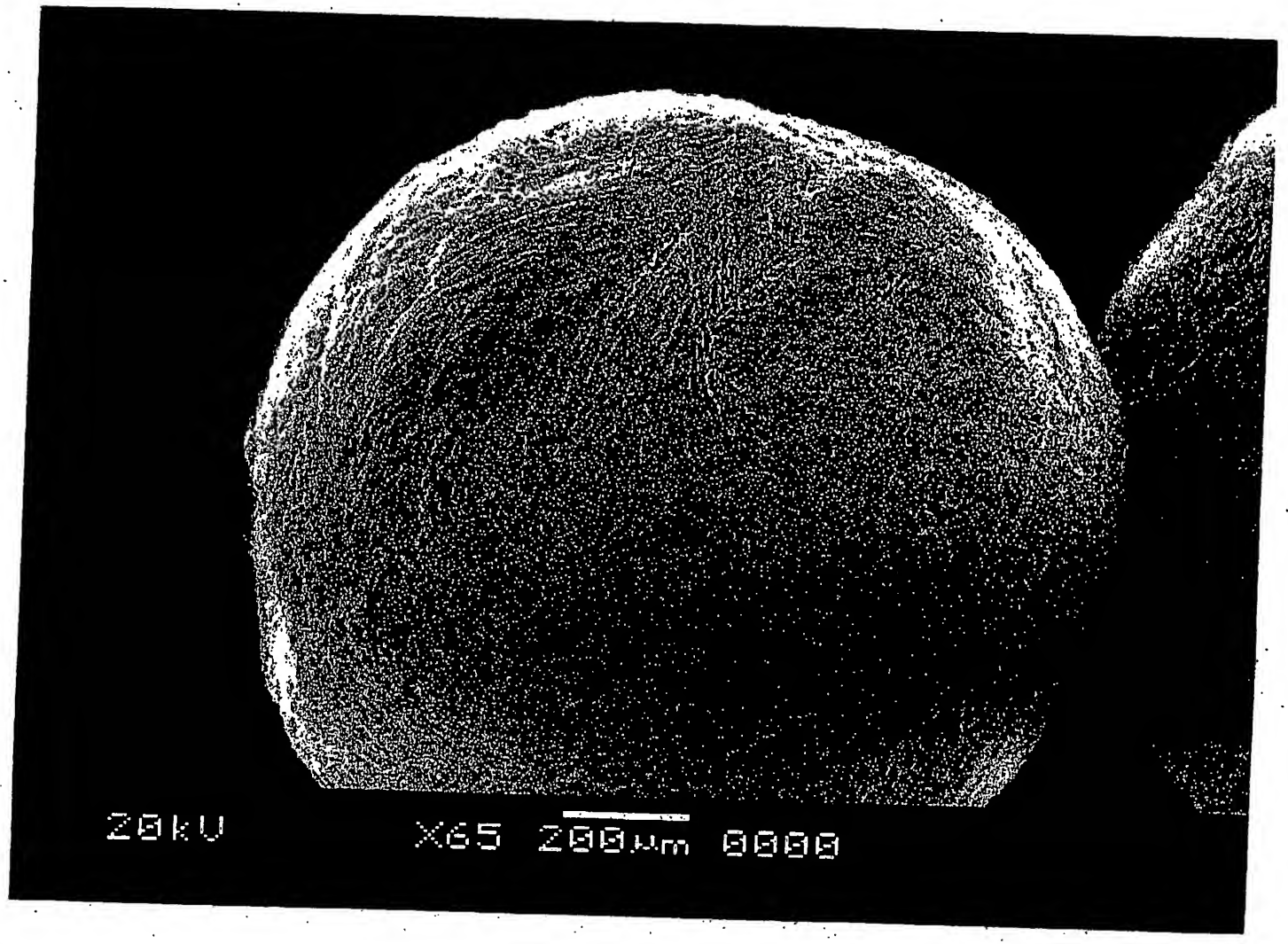
exposing the wetted cross-linked scaffold to a gradient of solvent mixtures comprising the non-aqueous solvent and water, starting with a high concentration of the non-aqueous solvent and ending with water.

ABSTRACT

The present invention relates to the development of new porous particulate collagen sponges, combining the desirable features of low toxicity, resorbability, and satisfactory porosity, particularly when wetted in an aqueous medium. Accordingly, the present invention is directed to new porous, particulate, dehydrothermally cross-linked, wetted sponges, as well as a process for making them.

Figure 1A

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 1B

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	

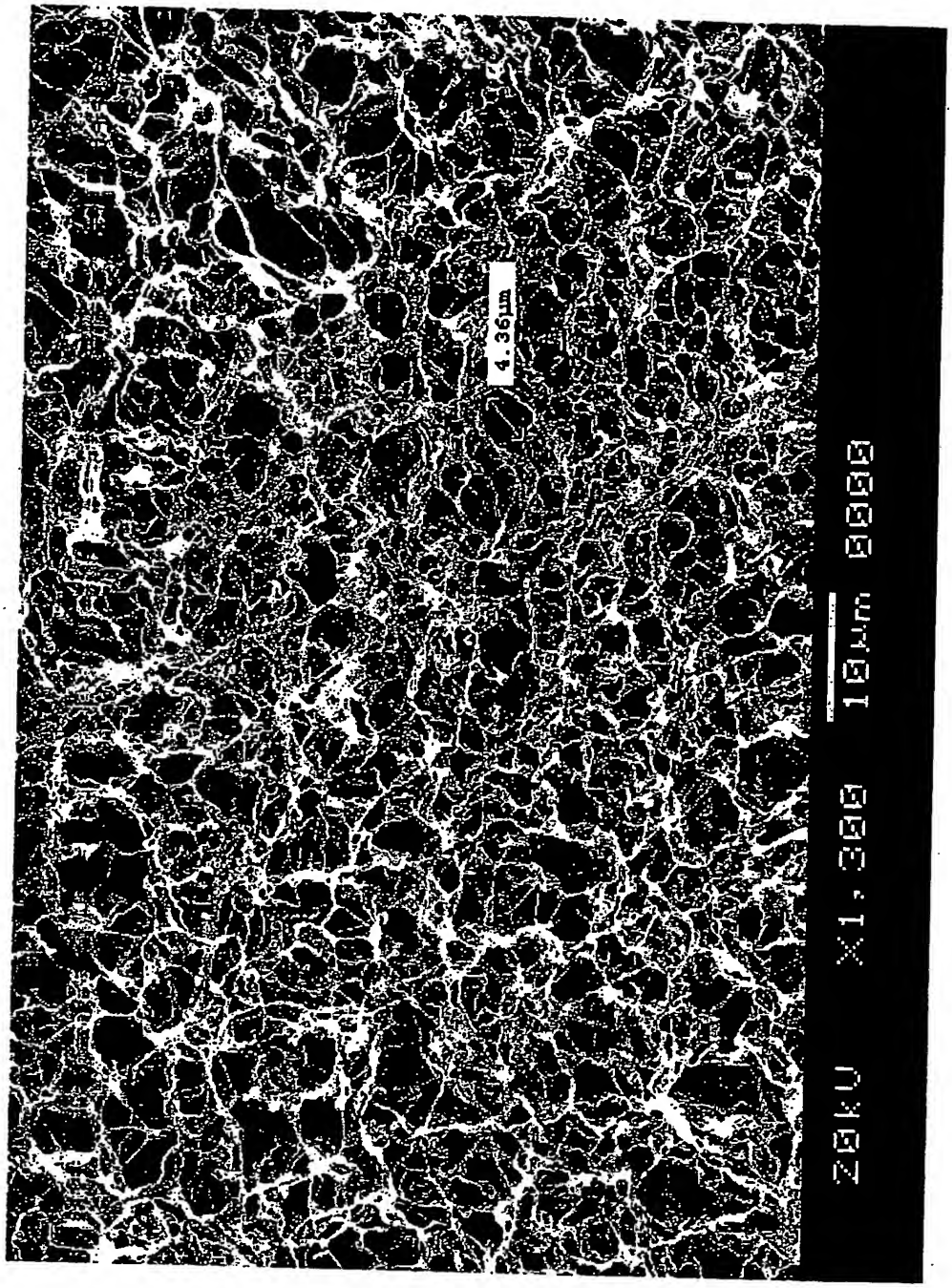


Figure 1C

Collagen Conc mg/ml
5

Acetic Acid Conc

Sample Shape
2mm to .5mm droplets

Cooling Conditions
Meter droplets of dispersion through no. 22 needle into coolant bath

Cooling Media
Liquid N2

Media Temp
C

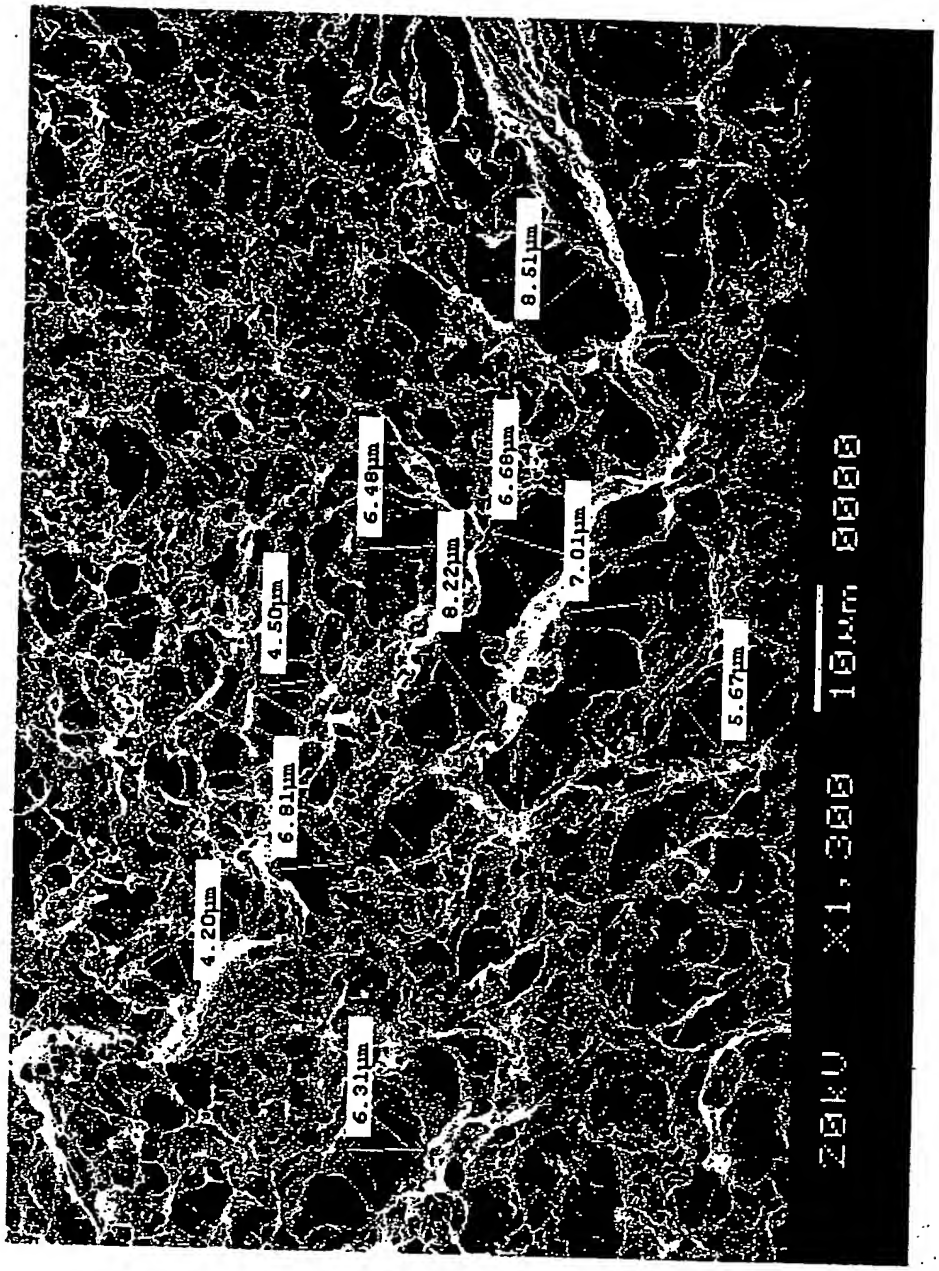


Figure 1C - 2nd Batch

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	

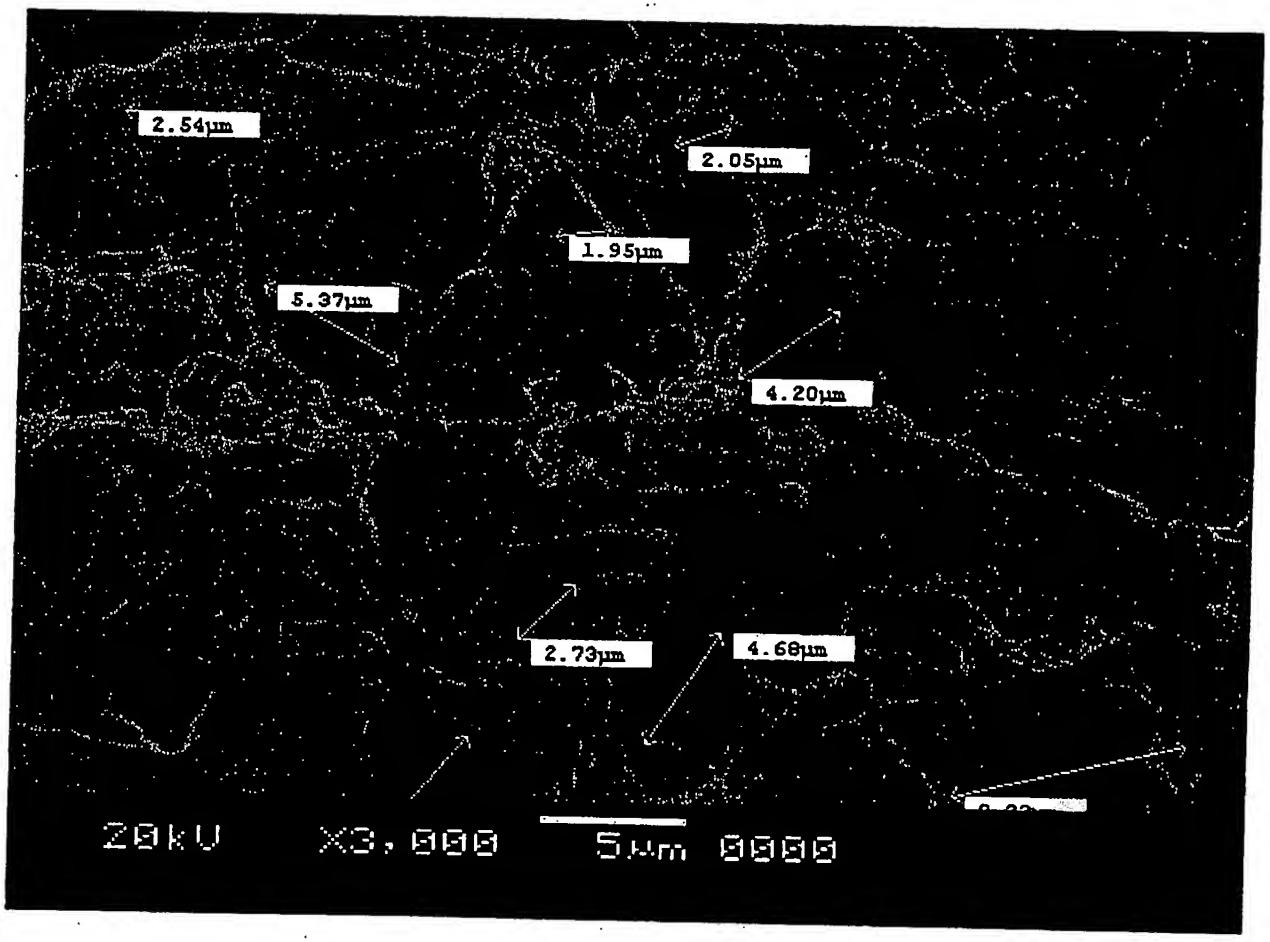


Figure 2

Collagen Conc mg/ml 3

Acetic Acid Conc %

Sample Shape

2mm to .5mm droplets

Cooling Conditions

Meter droplets of dispersion through no. 22 needle into coolant bath

Cooling Media

Liquid N2

Media Temp C

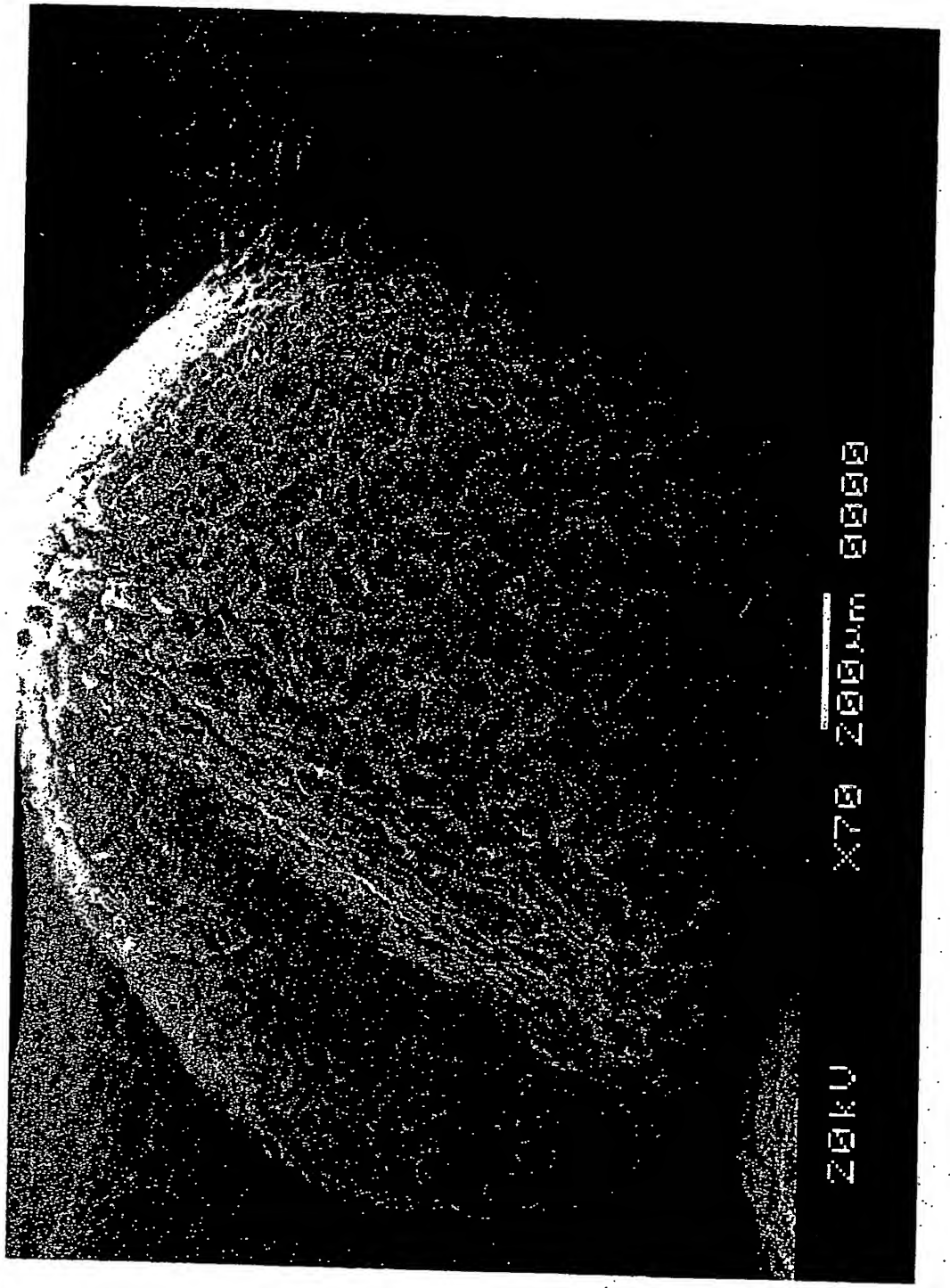


Figure 2A

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
3		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	

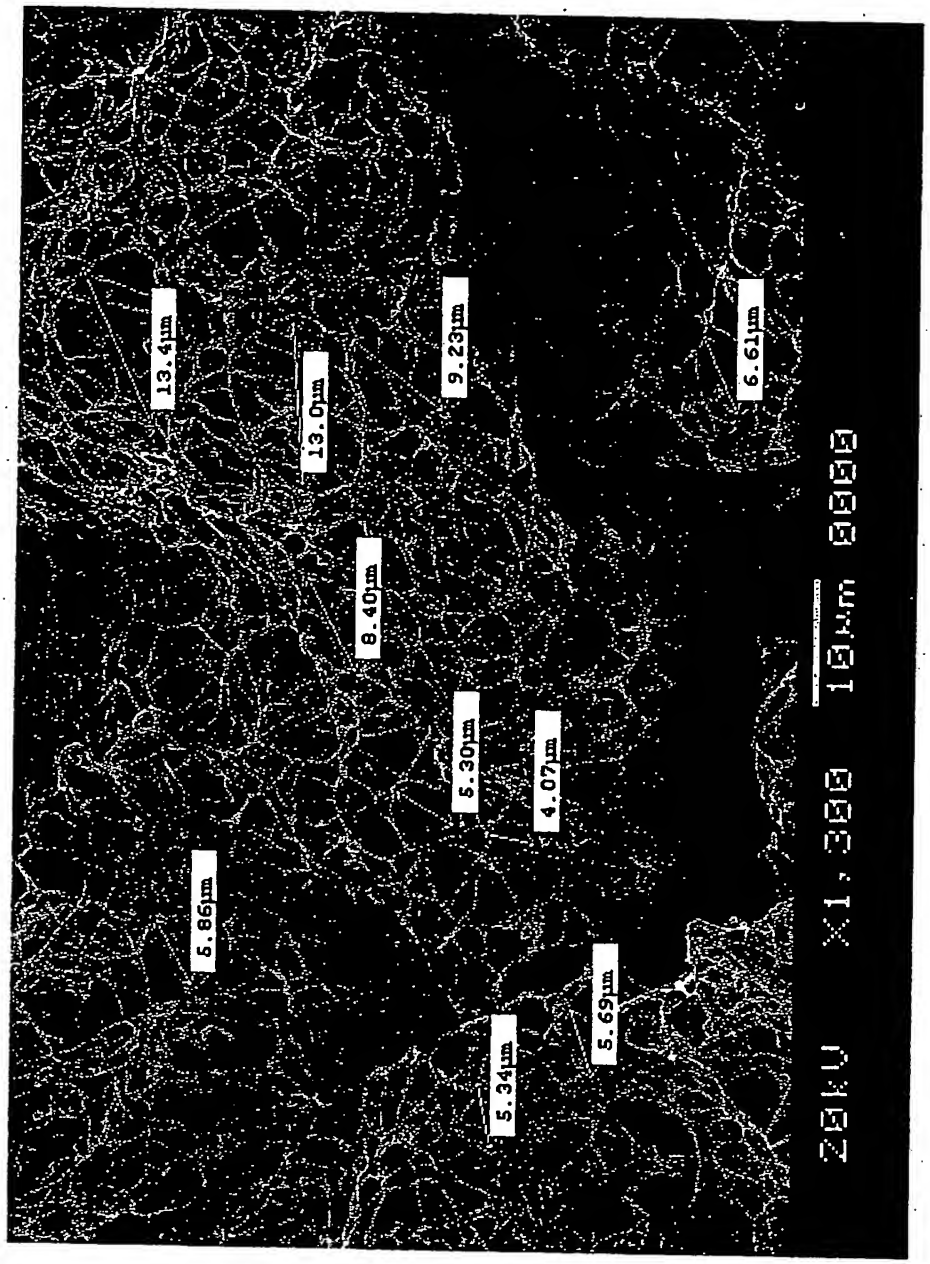
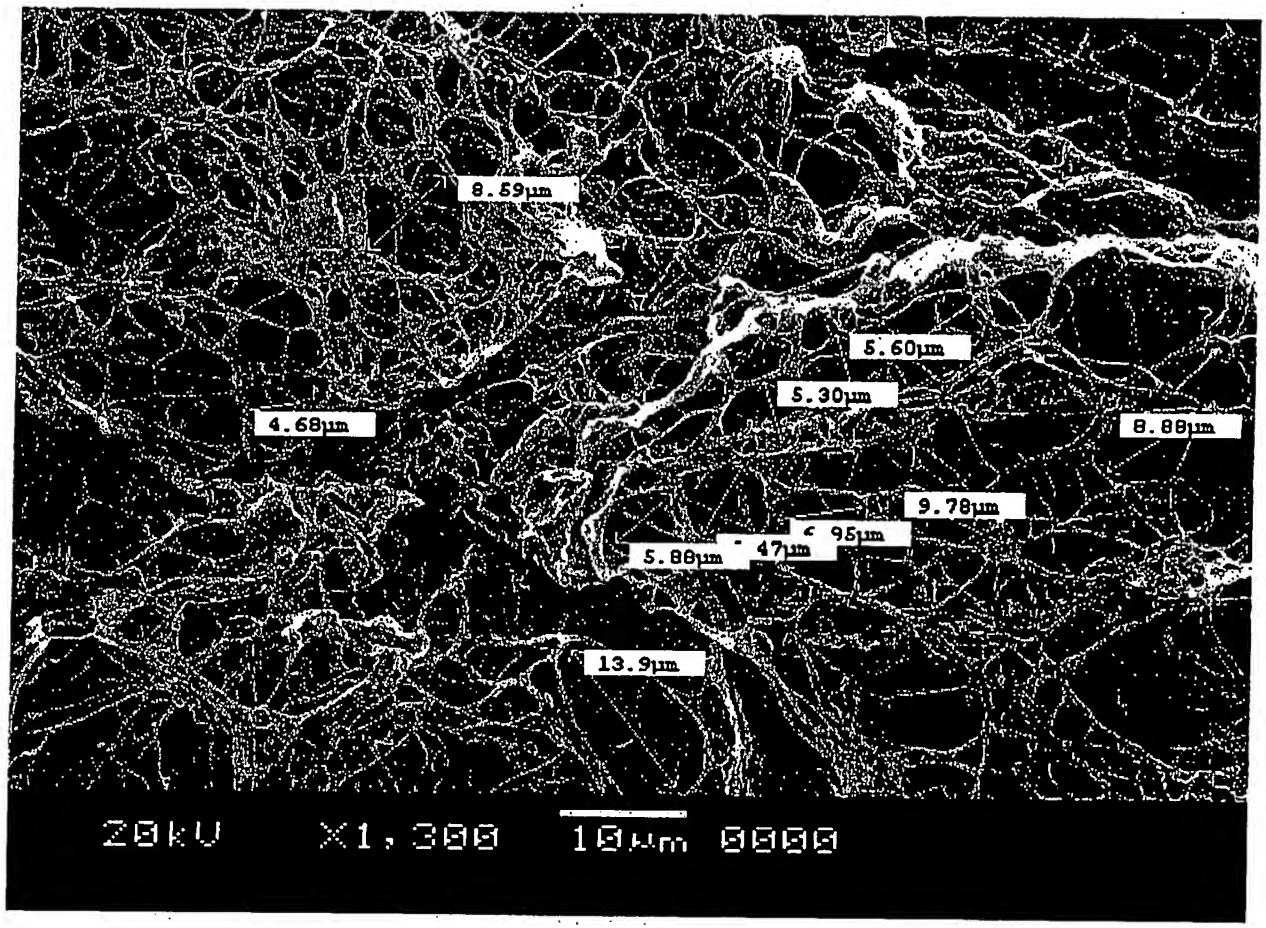


Figure 2B

Collagen Conc. mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
3		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

Docket No.: GRN-005-2

Figure 3

Collagen Conc mg/ml	Acetic Acid Conc.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
1		2mm to .5mm droplets	Meter droplets of dispersion through no: 22 needle into coolant bath	Liquid N2	

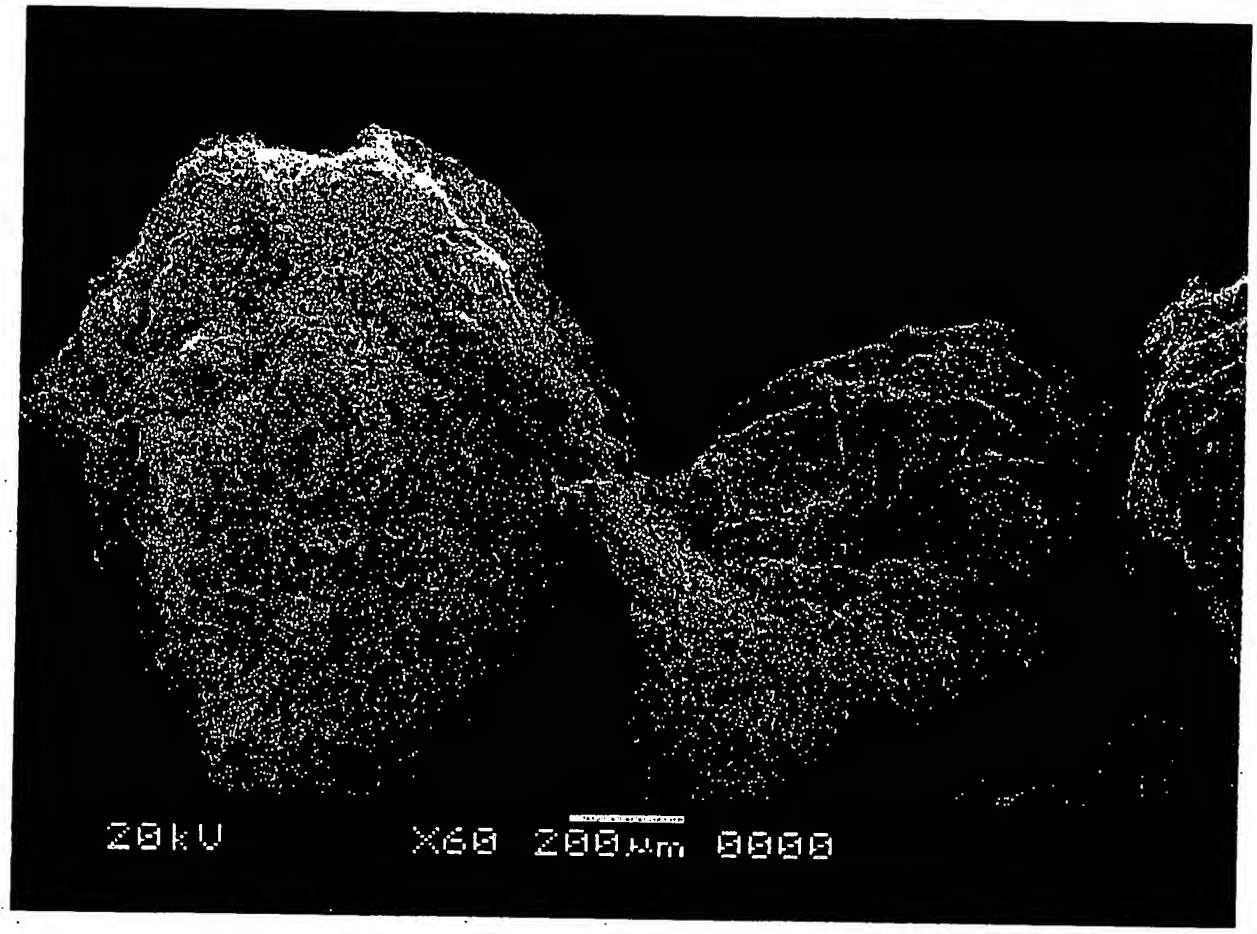


Figure 3A

Collagen Conc mg/ml: 1

Acetic Acid Conc:

Sample Shape: 2mm to .5mm droplets

Cooling Conditions: Meter droplets of dispersion through no. 22 needle into coolant bath

Cooling Media: Liquid N2

Media Temp C:

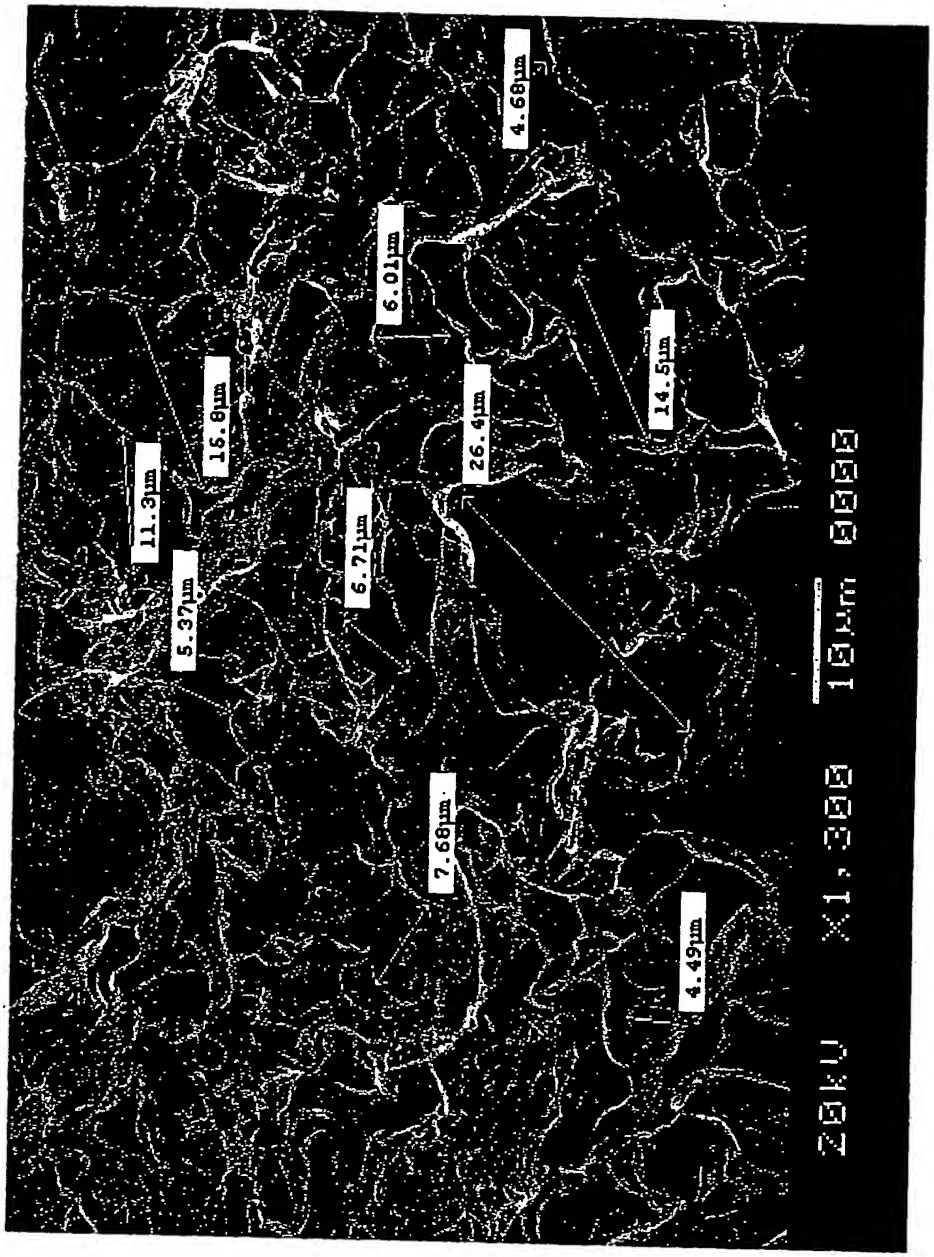


Figure 3B

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
1		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	Liquid N2	

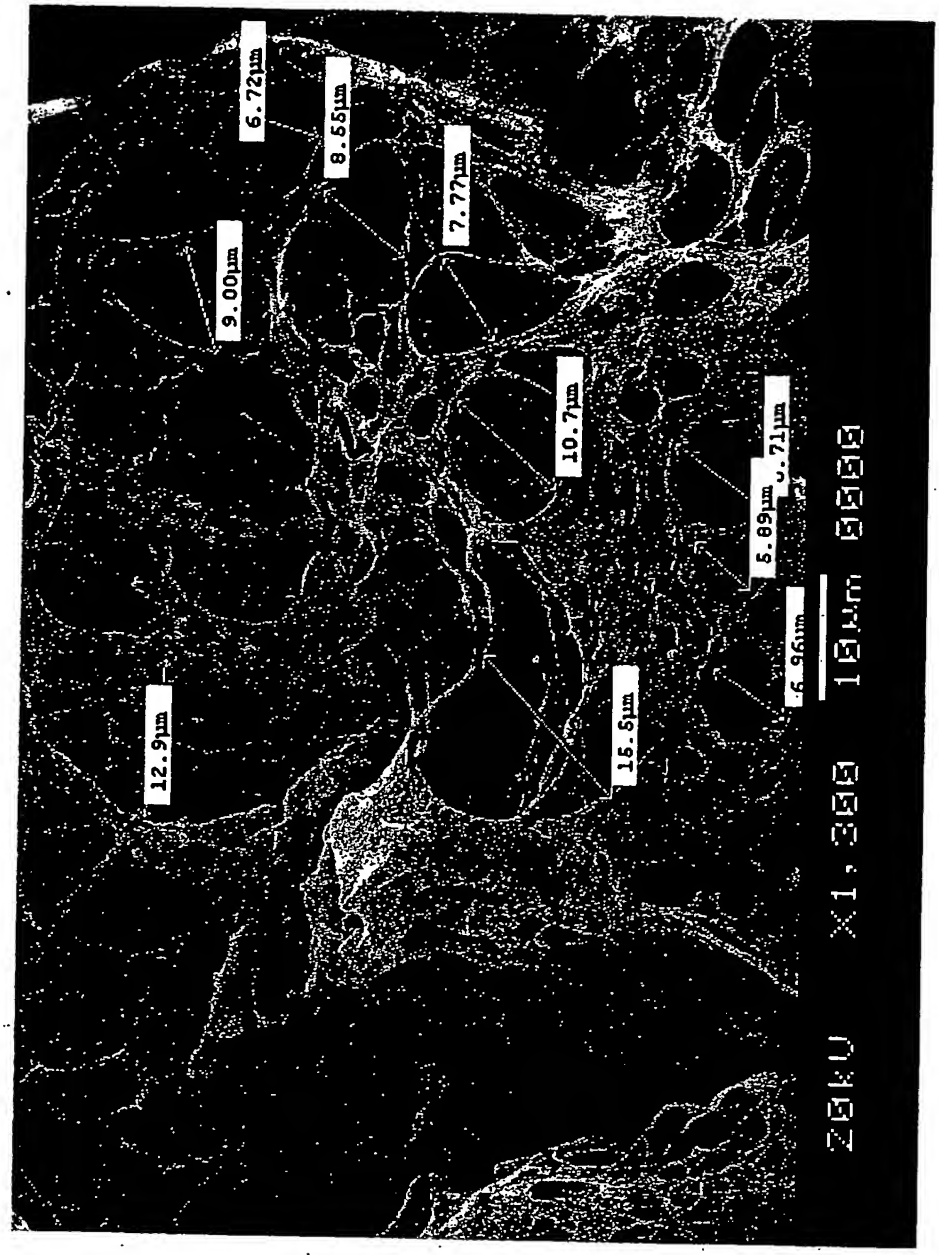


Figure 4

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

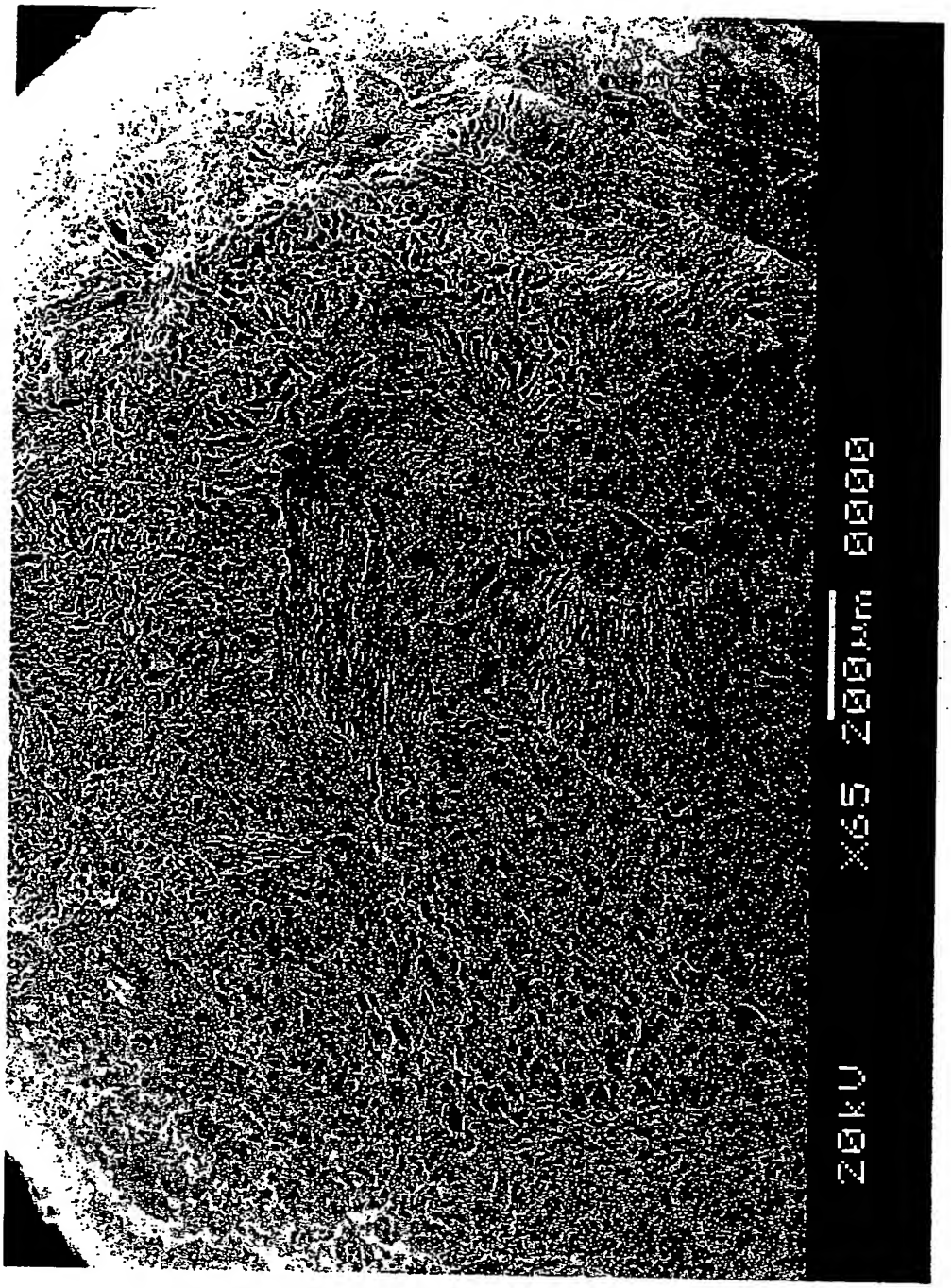


Figure 4A

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

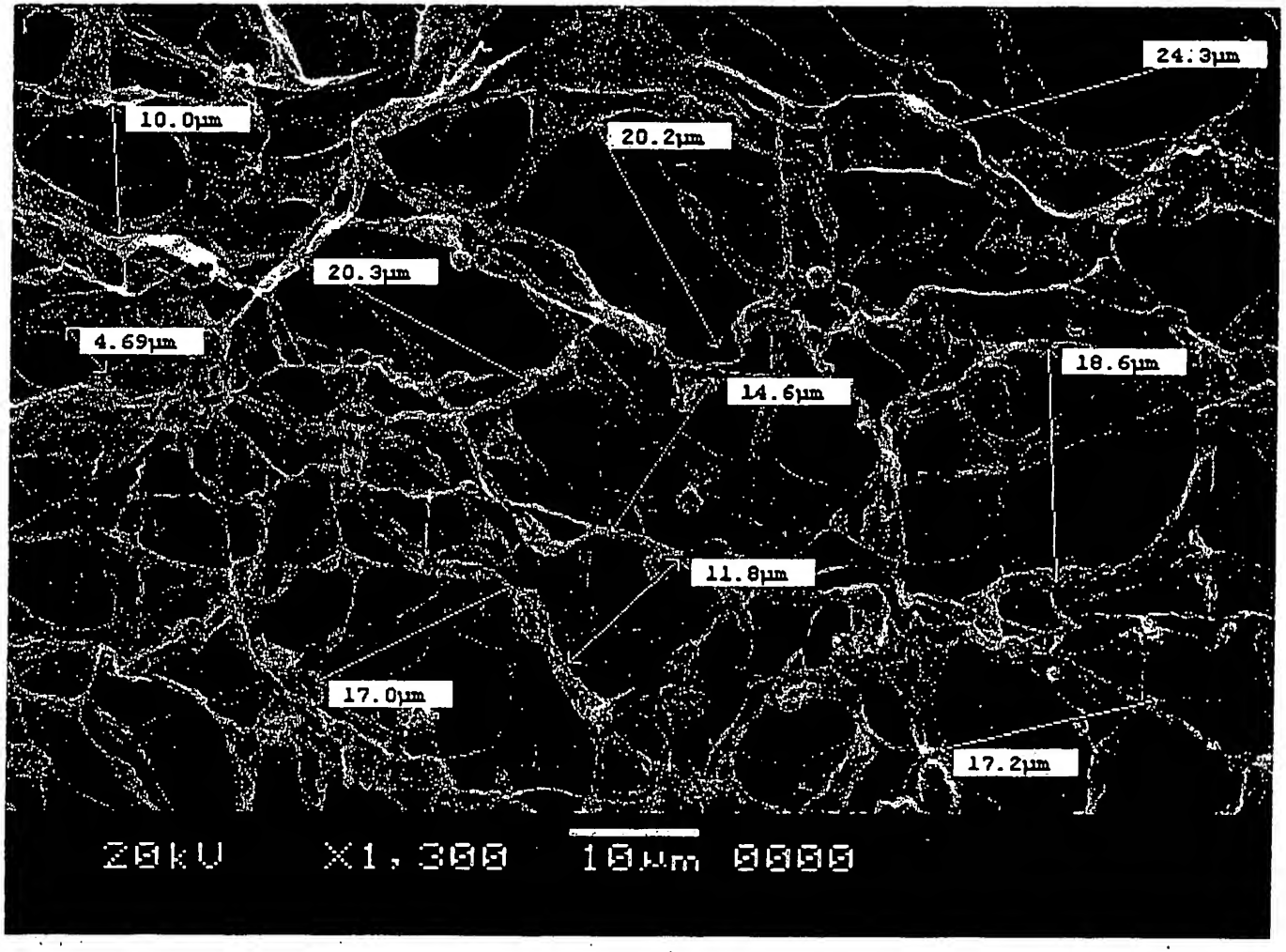


Figure 4B

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

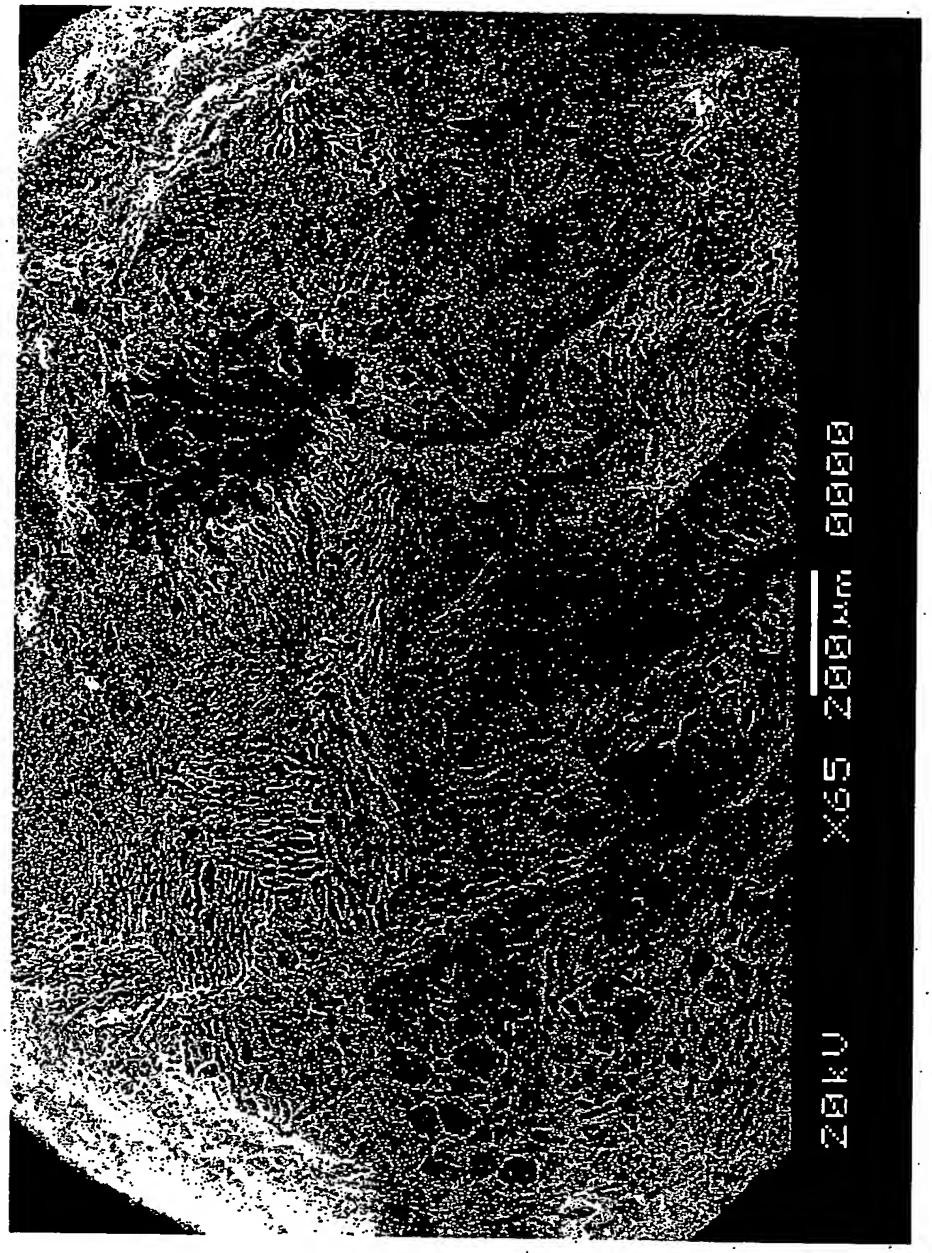
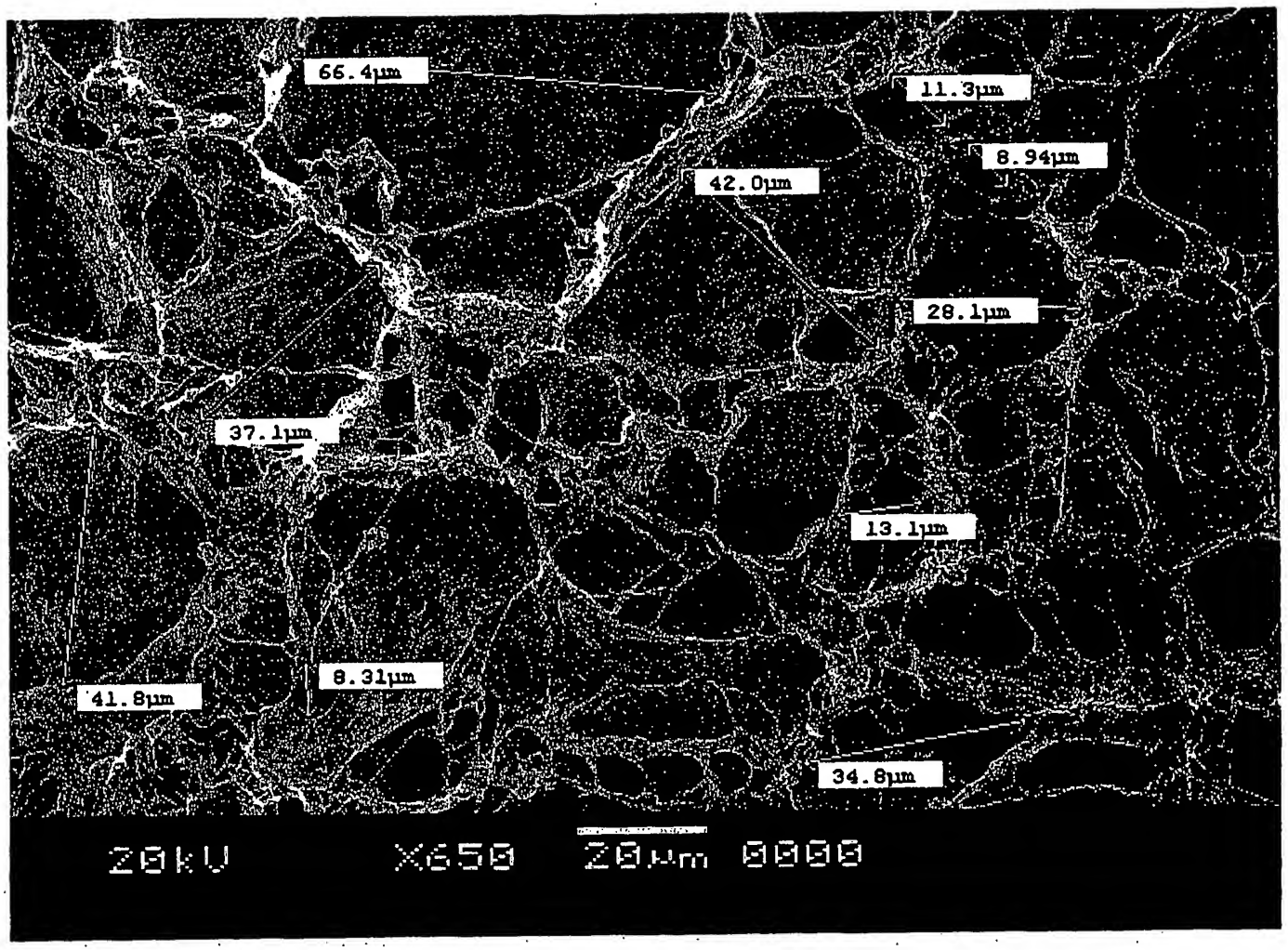


Figure 4C

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

Docket No.: GRN-005-2

Figure 4D

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

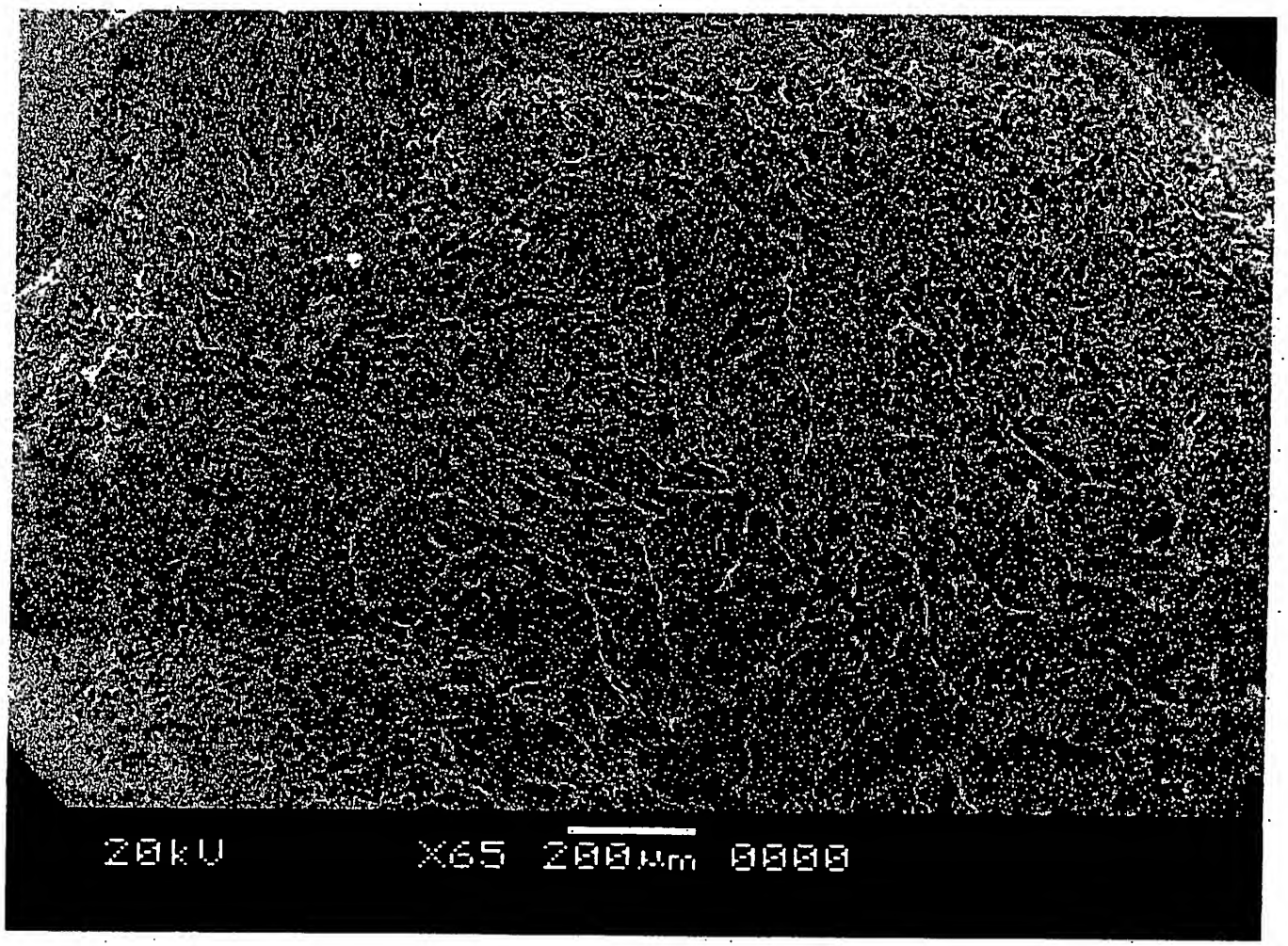


Figure 4E

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

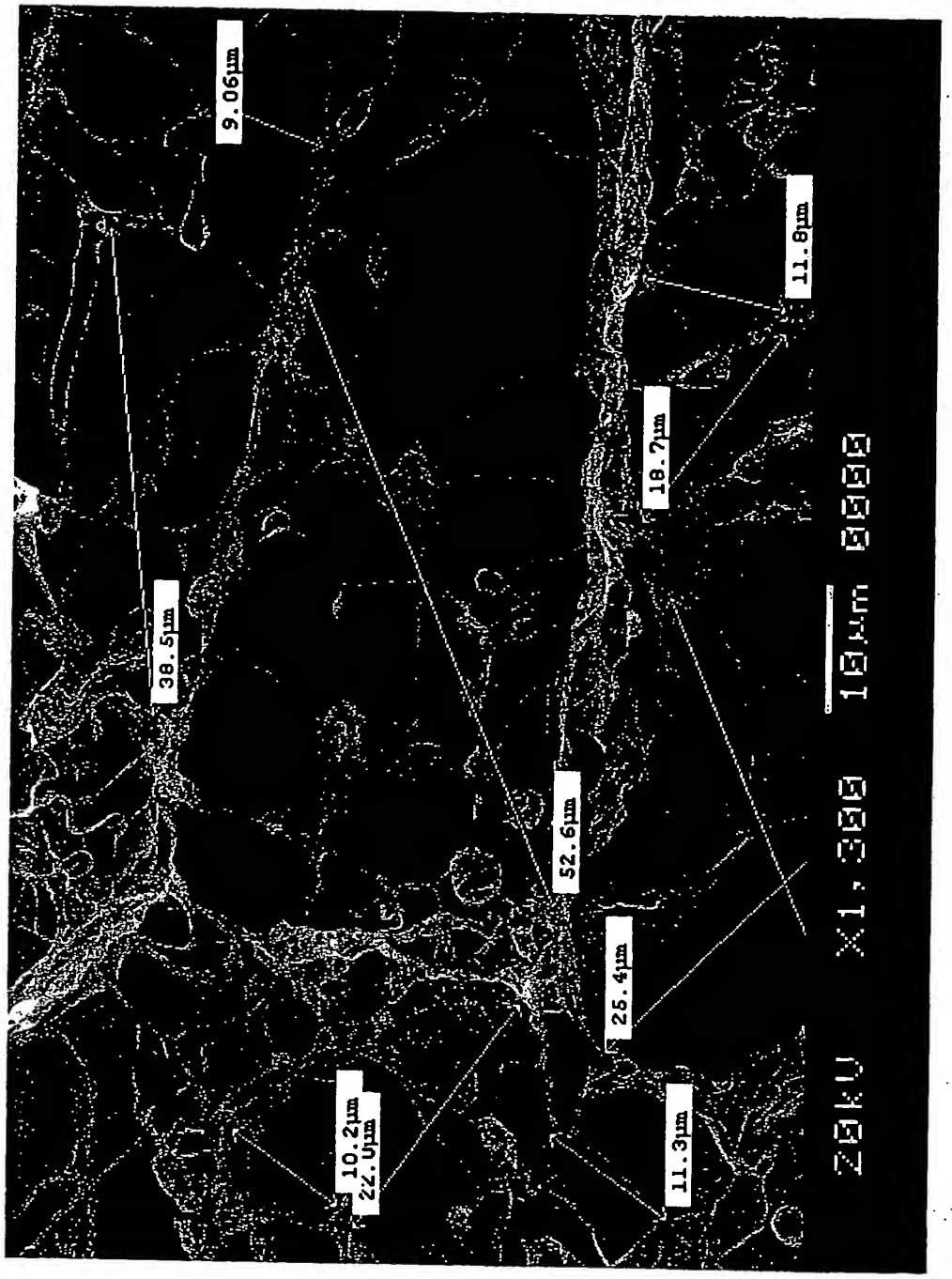


Figure 4 - 2nd Batch

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

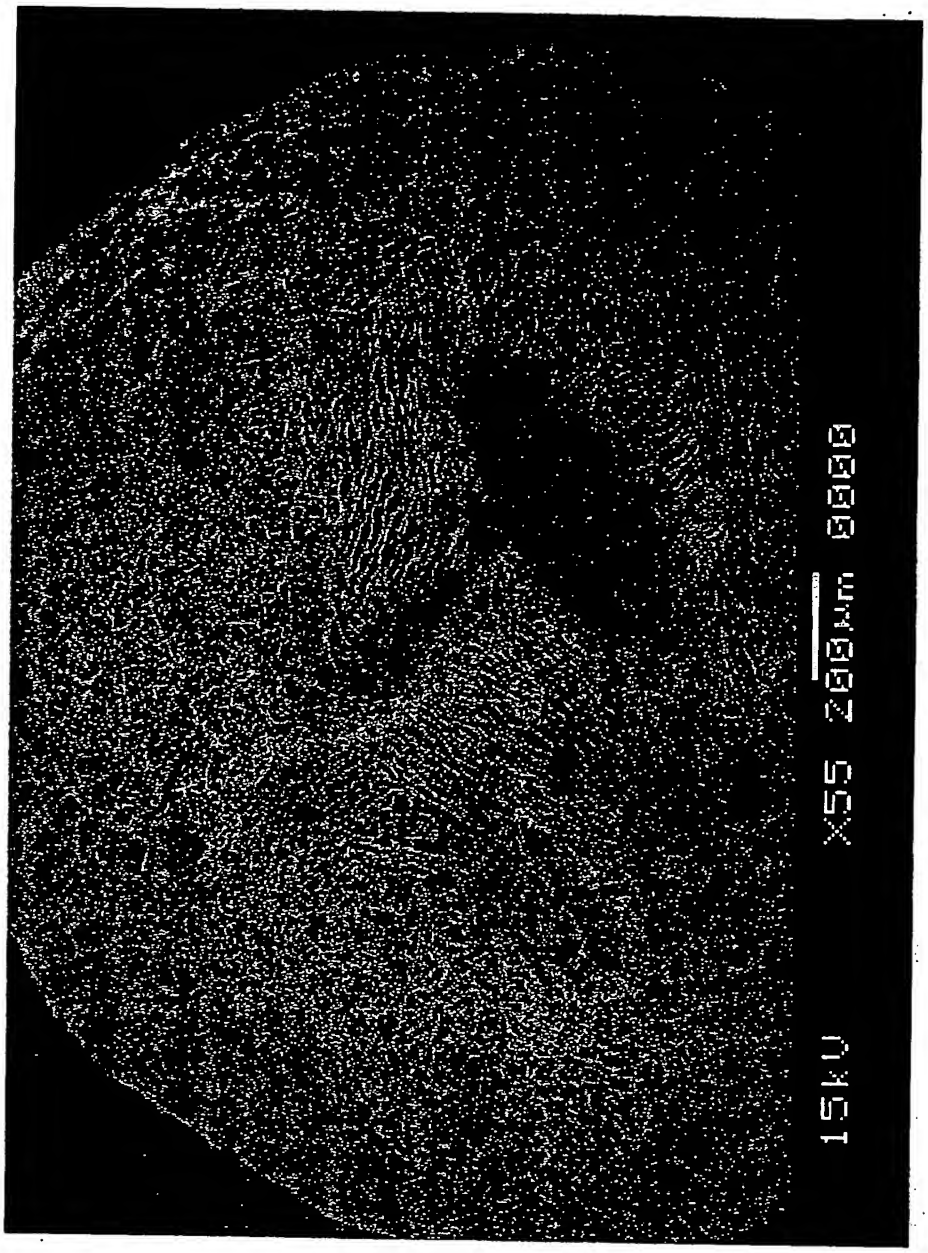


Figure 4A - 2nd Batch

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

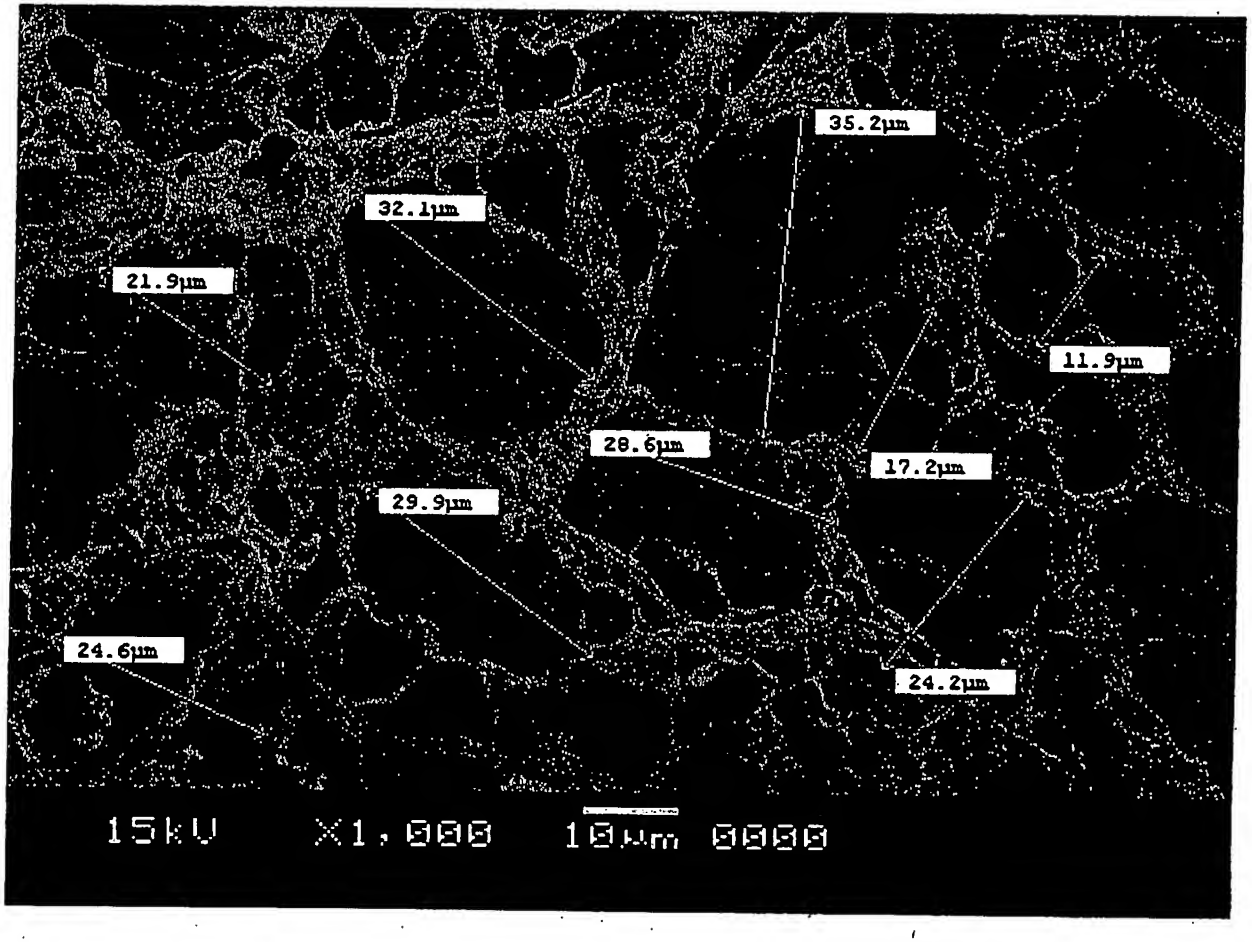


Figure 4B - 2nd Batch

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

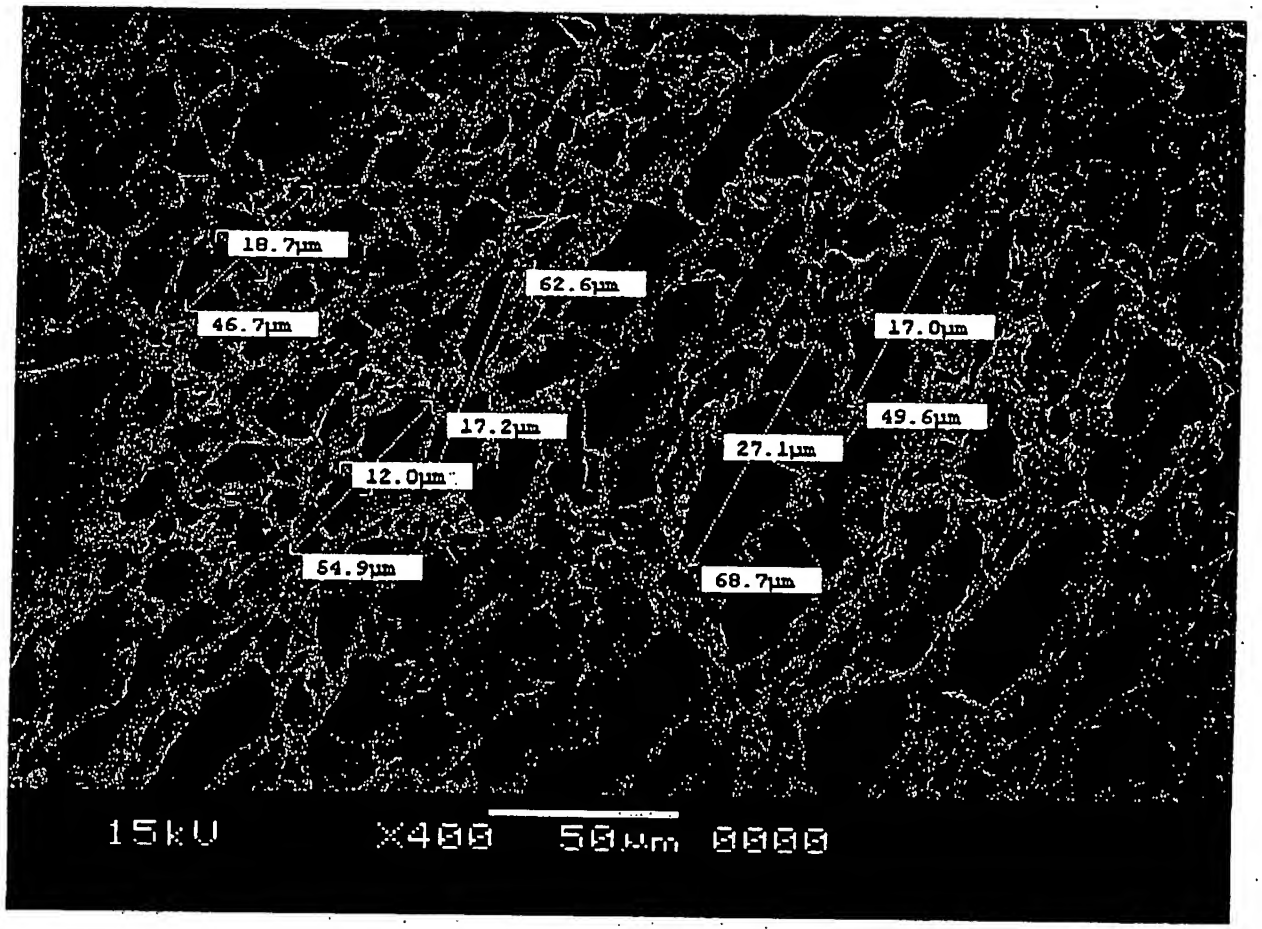
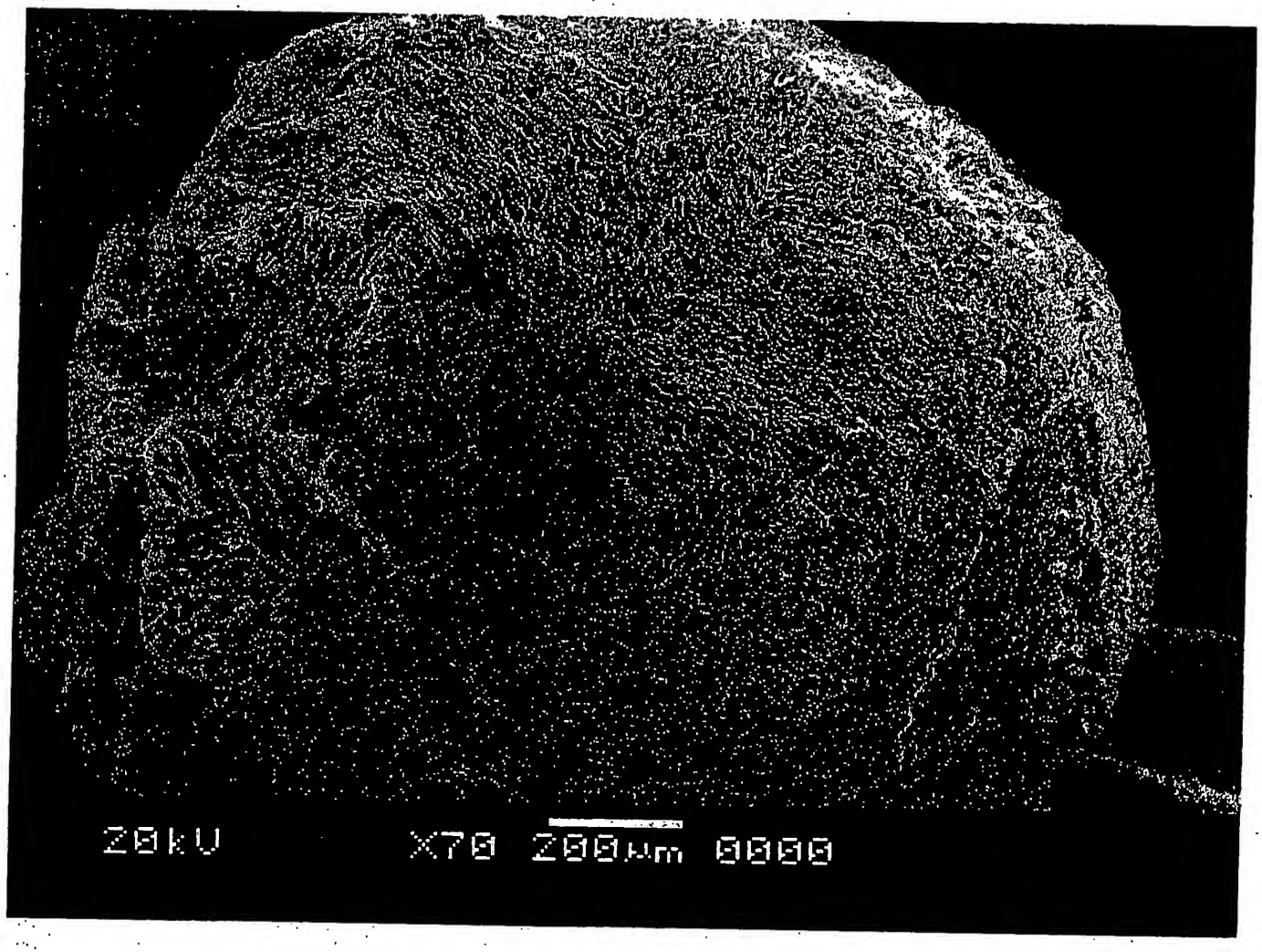


Figure 5

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
3		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 5A

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
3		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

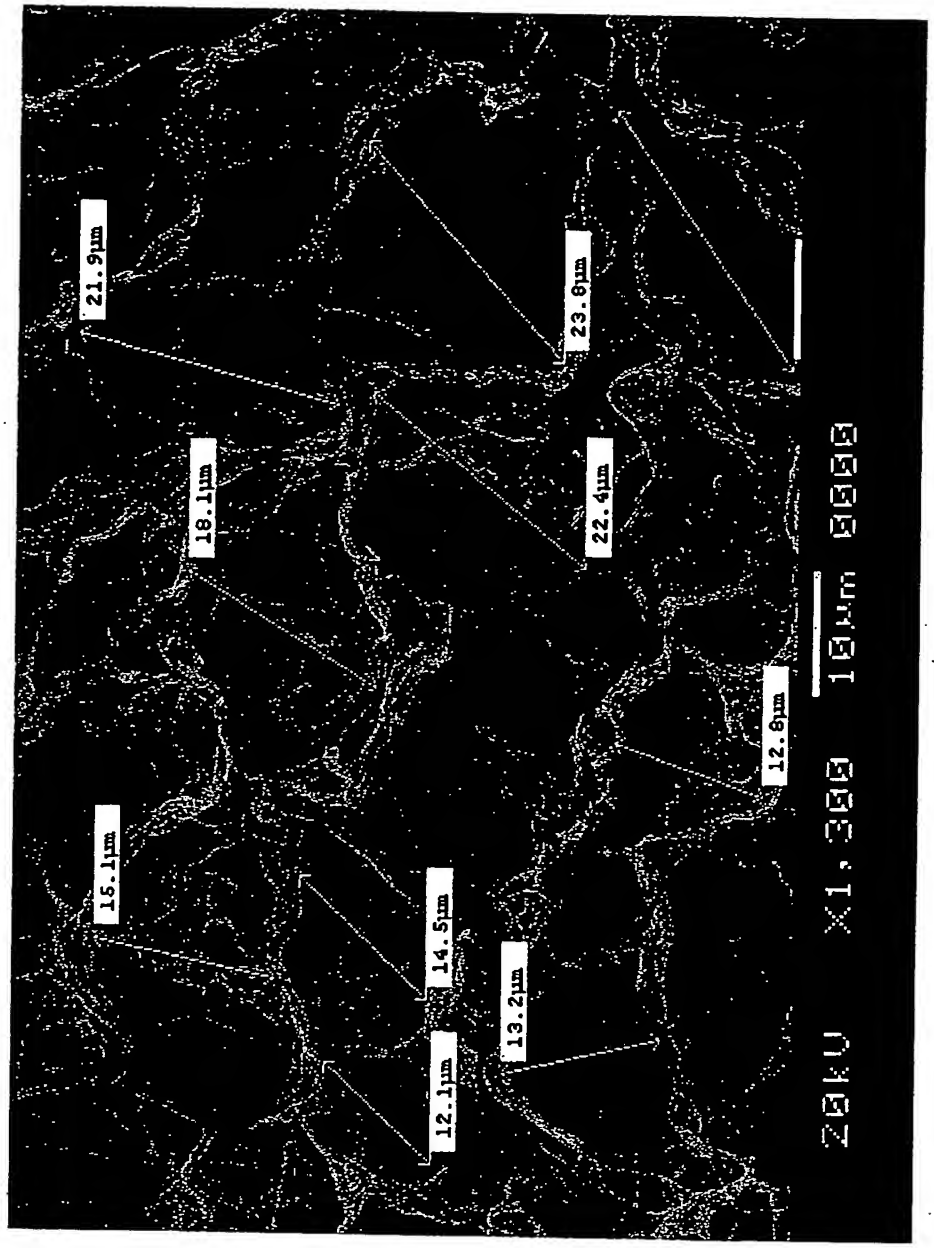


Figure 5B

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
3		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20

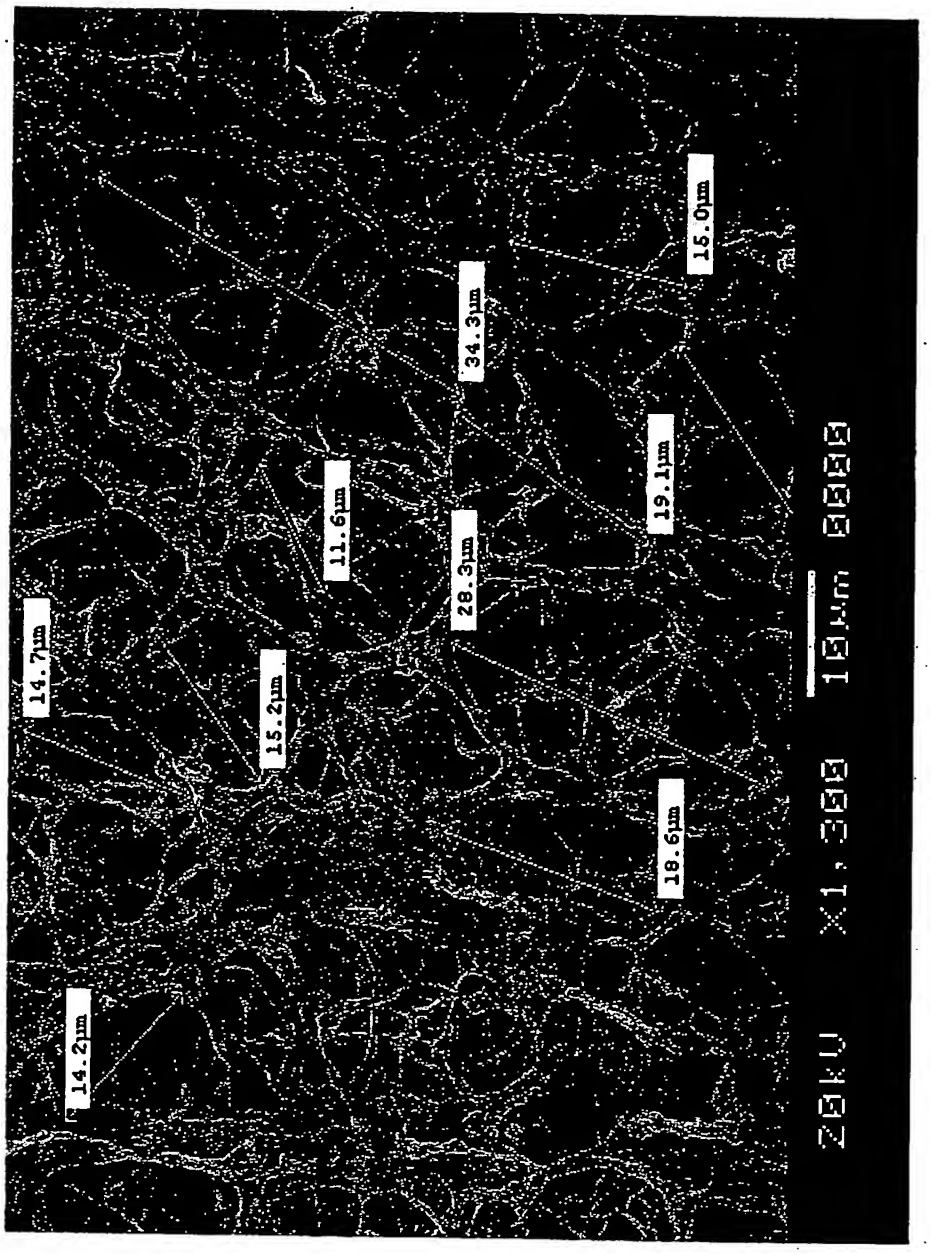
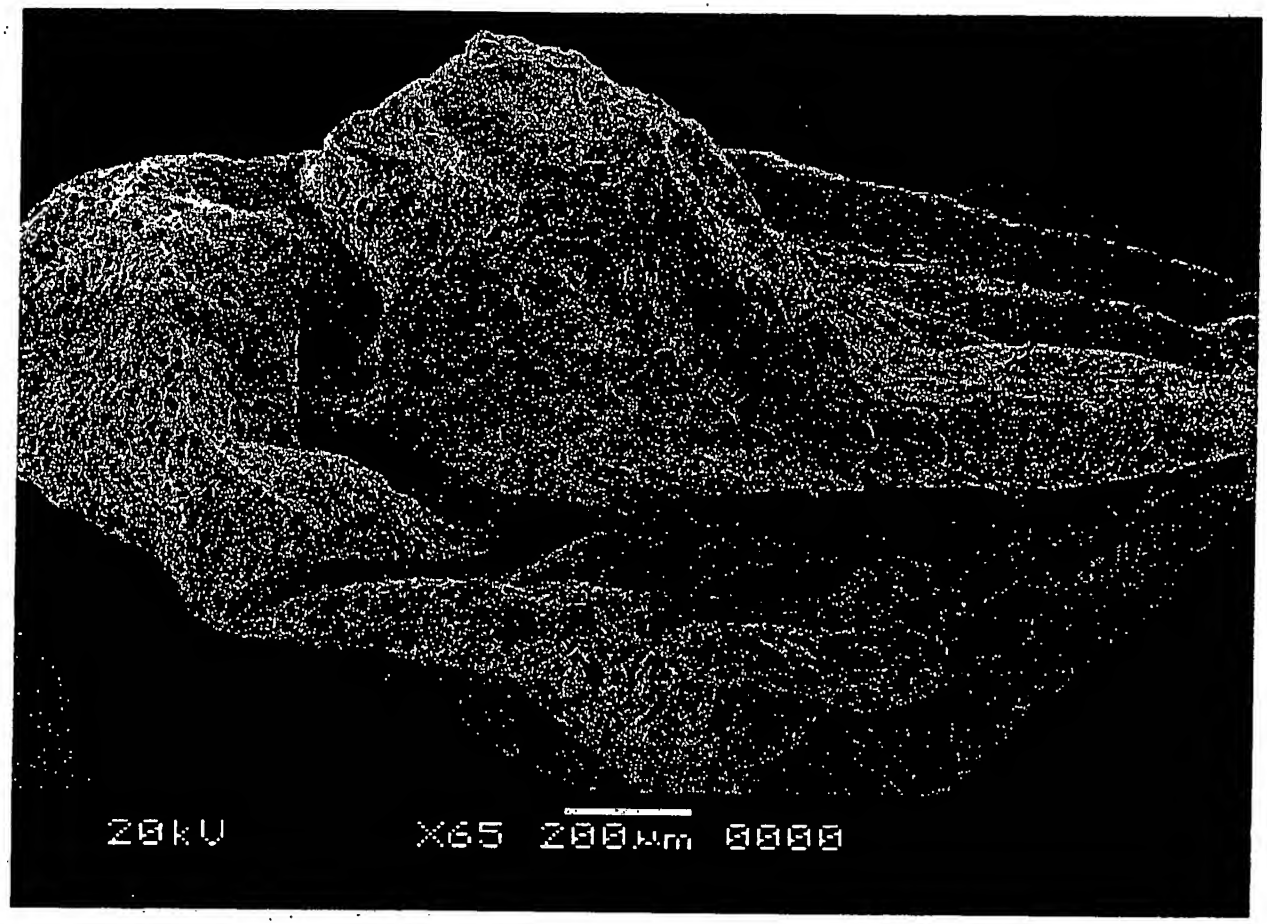


Figure 6

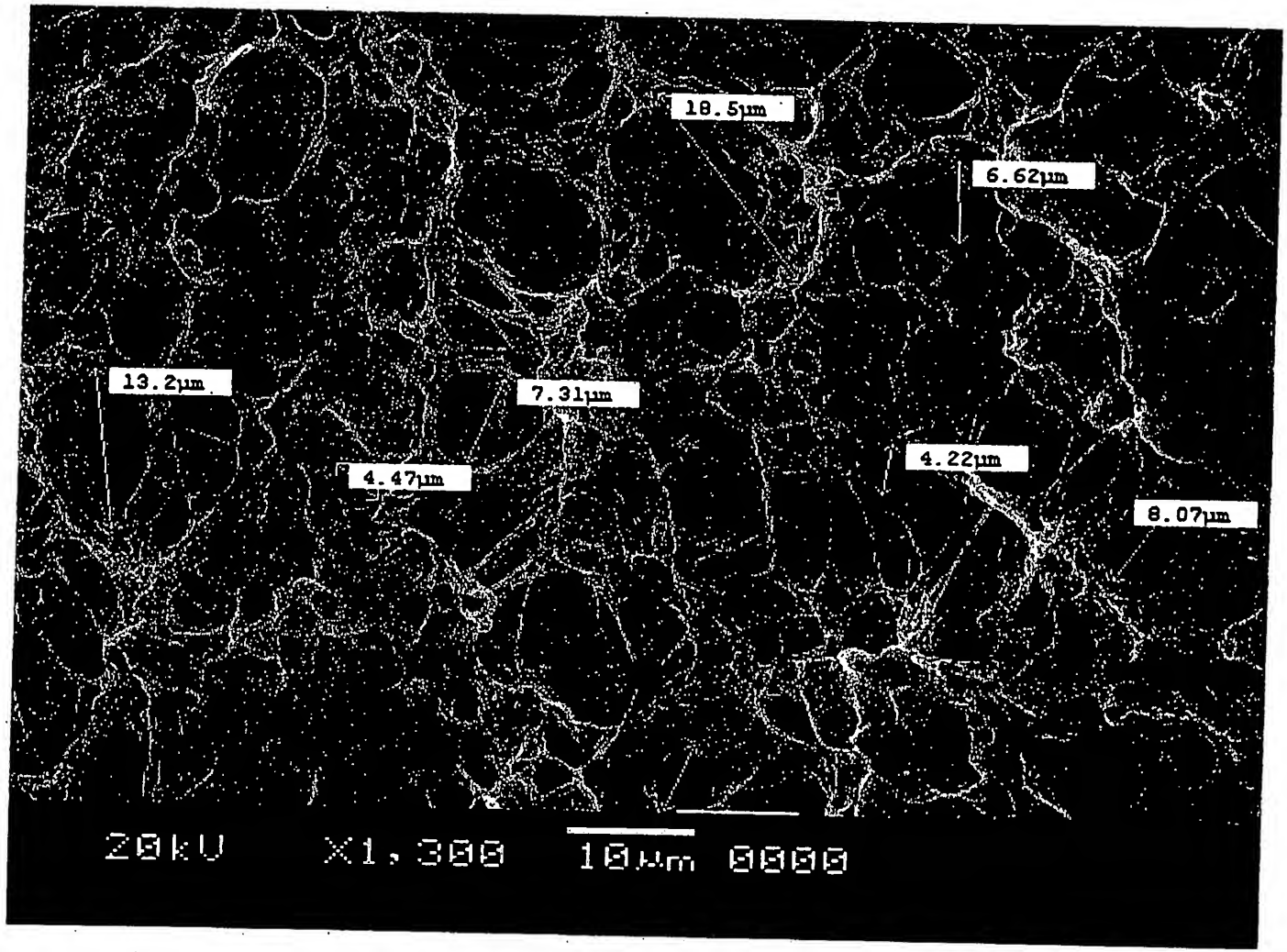
Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
1		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20	



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 6A

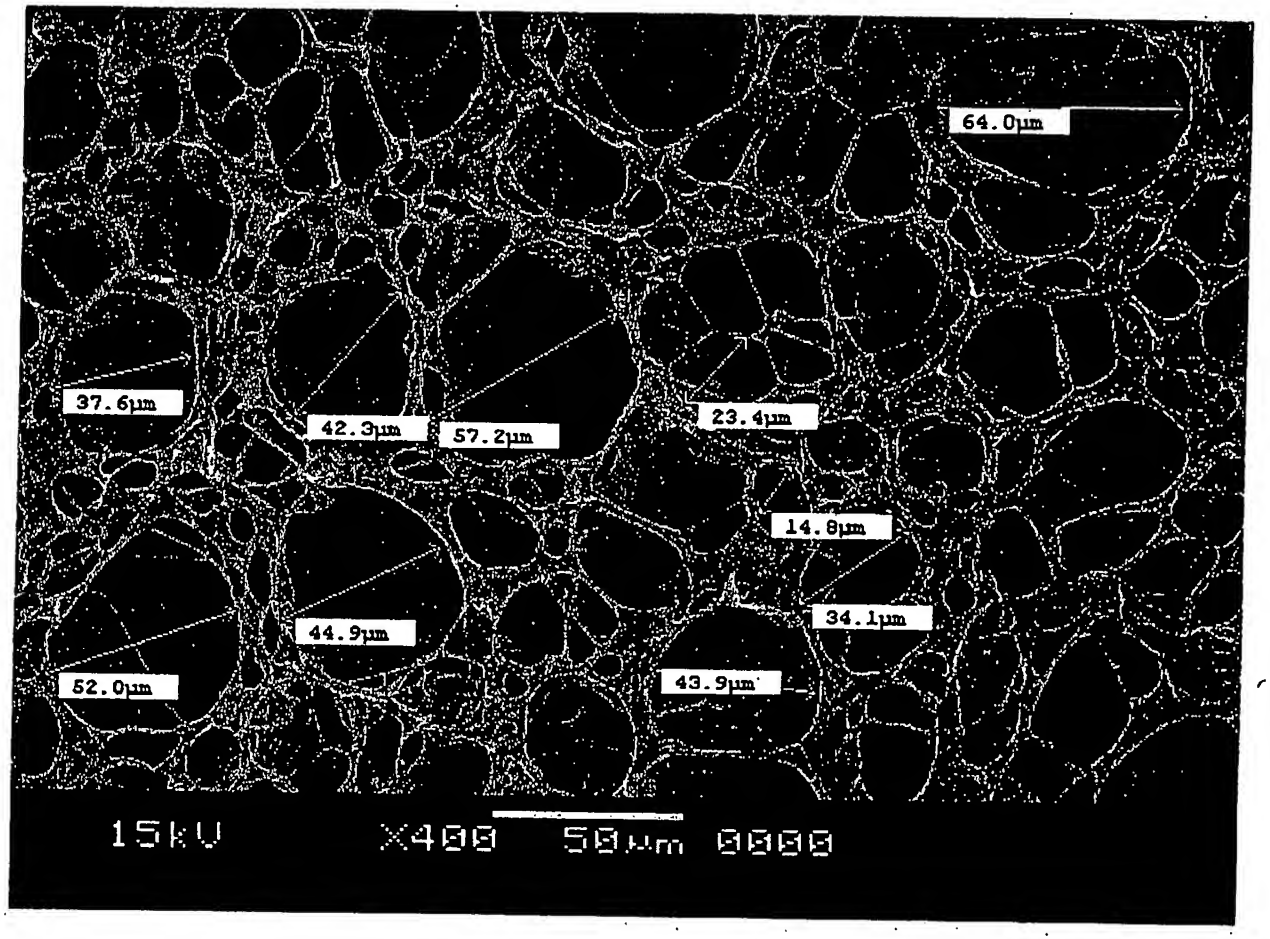
Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
1		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-20	



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 7

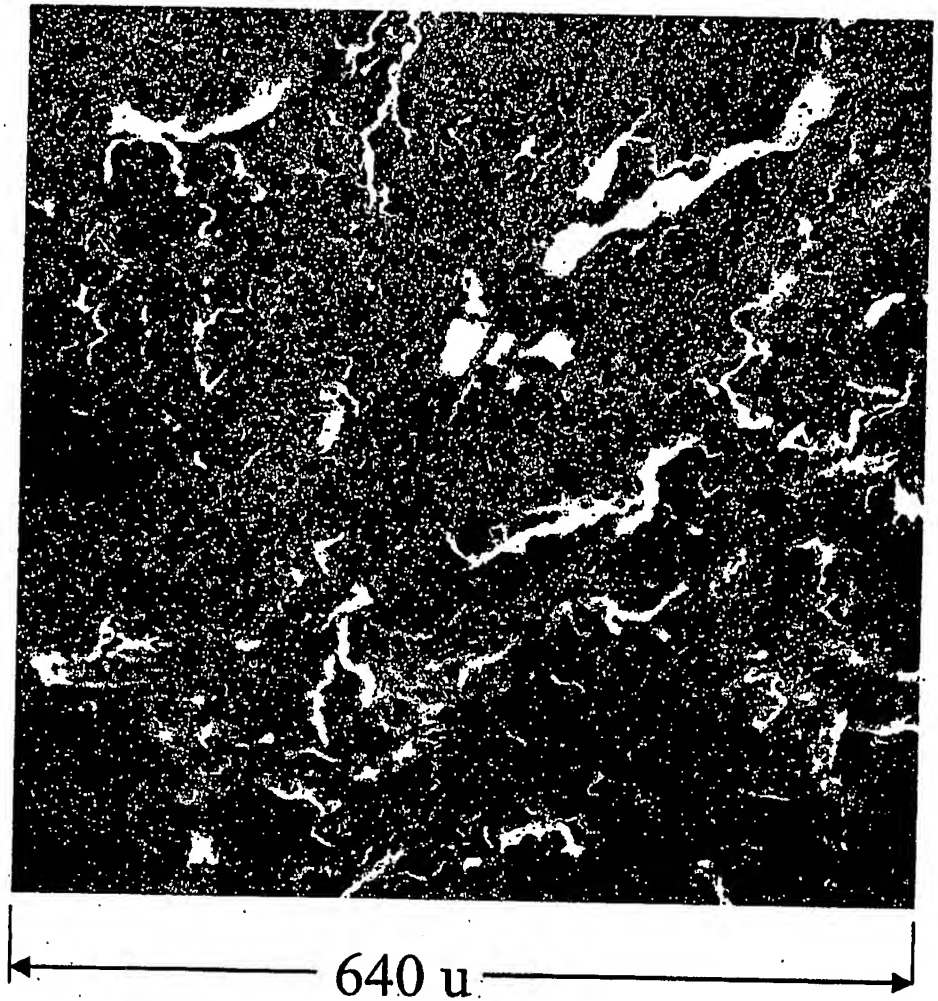
Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp	Wetting Method
3	droplet placed on release sheet	At room temp., place droplets on to release lines. Place assembly in insulate container. Place container in -20C freezer	air	-20	series



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 9

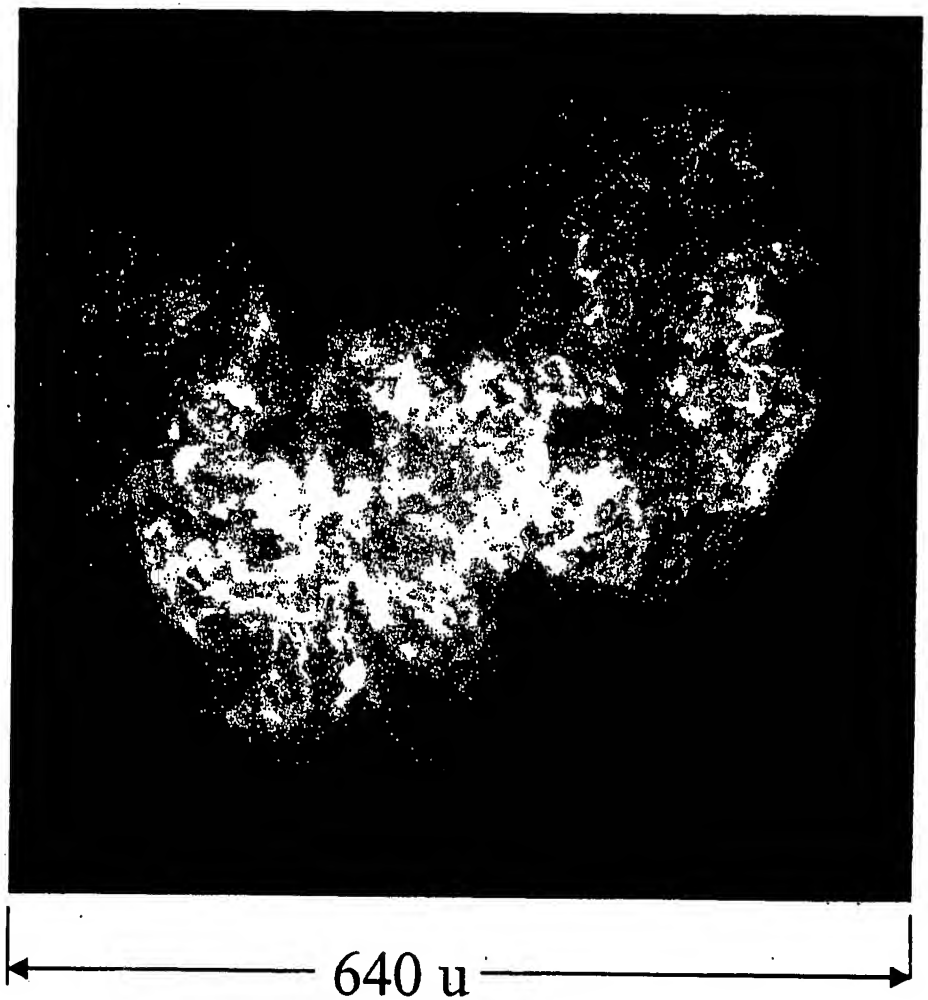
Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
1	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into	Liquid N2		series



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 10

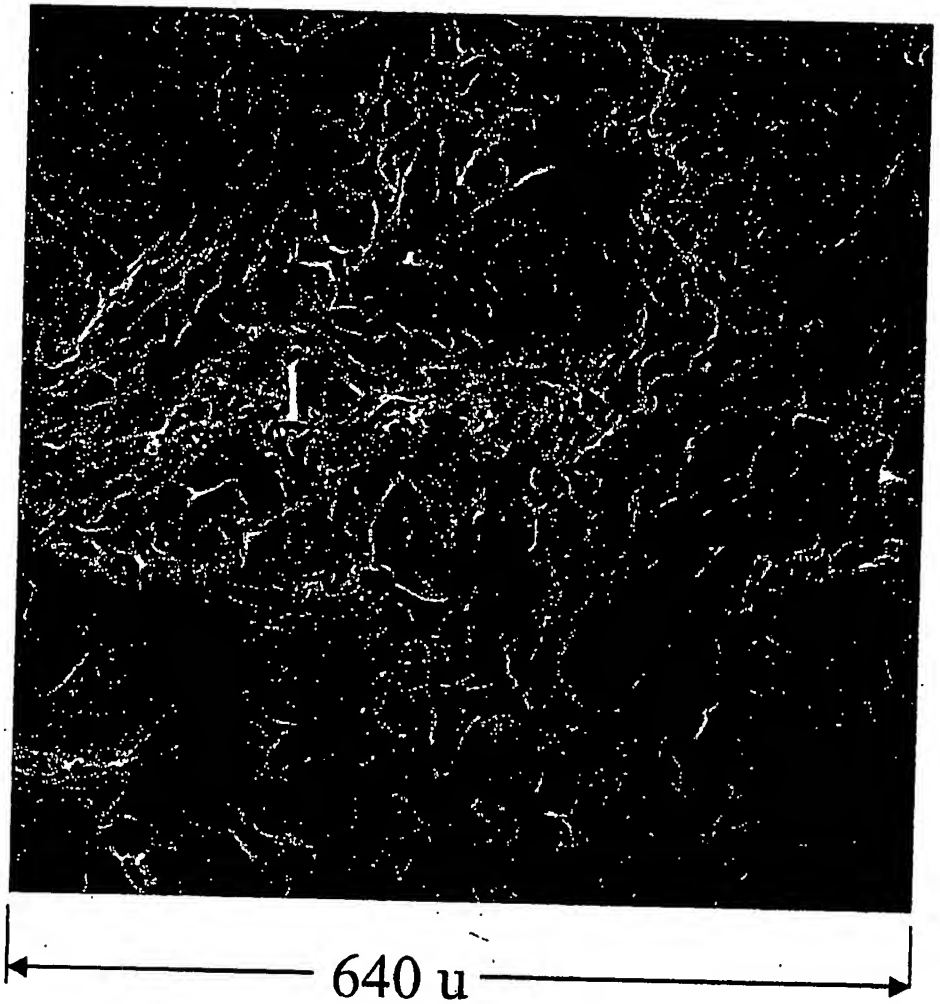
Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
1	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into	Liquid N2		direct



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 11

Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
2	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into	pentane	-15	series



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 12

Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C	Wetting Method
2	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into	pentane	-15	direct

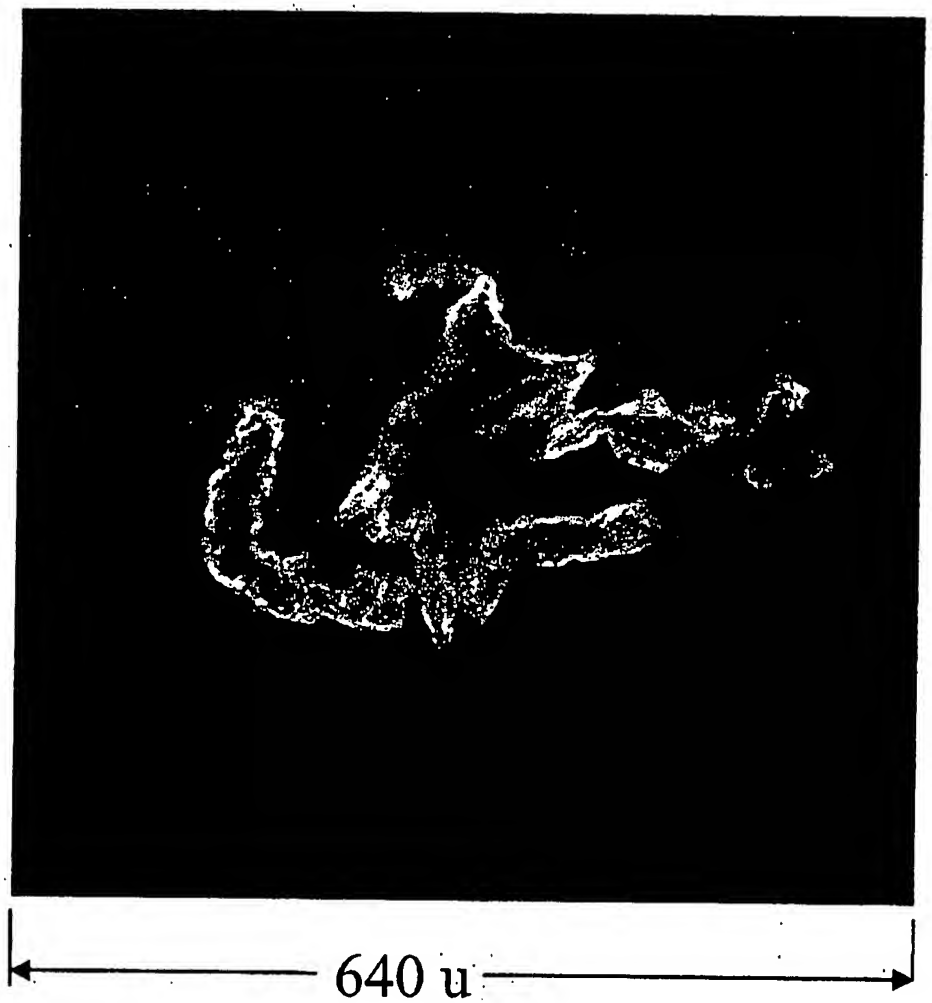


Figure 13

Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp	Wetting Method
3	droplet placed on release sheet	At room temp., place droplets on to release lines. Place assembly in insulate container. Place container in -20C freezer	air	-20	series

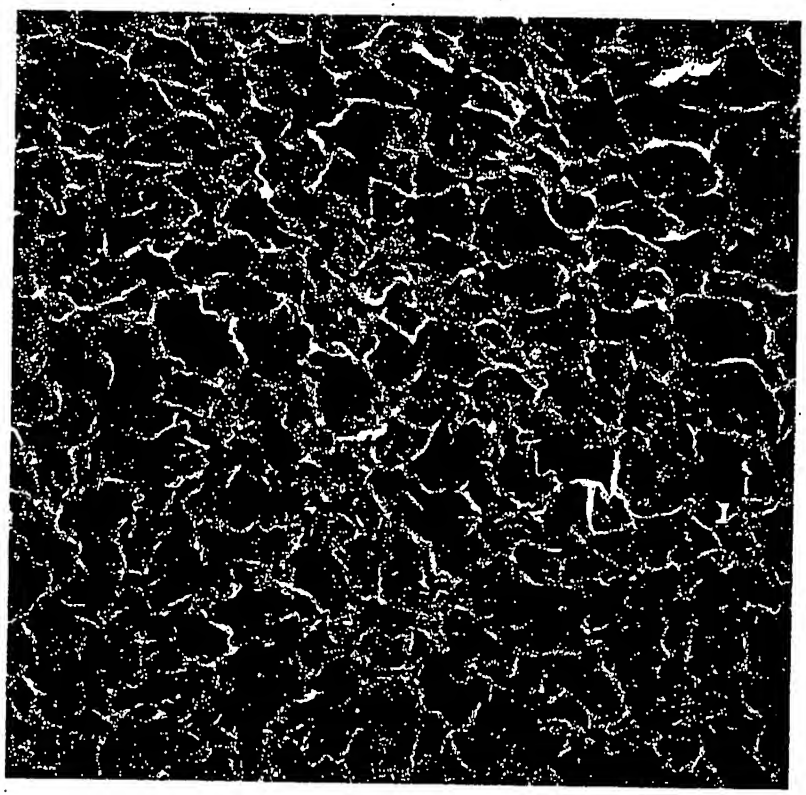
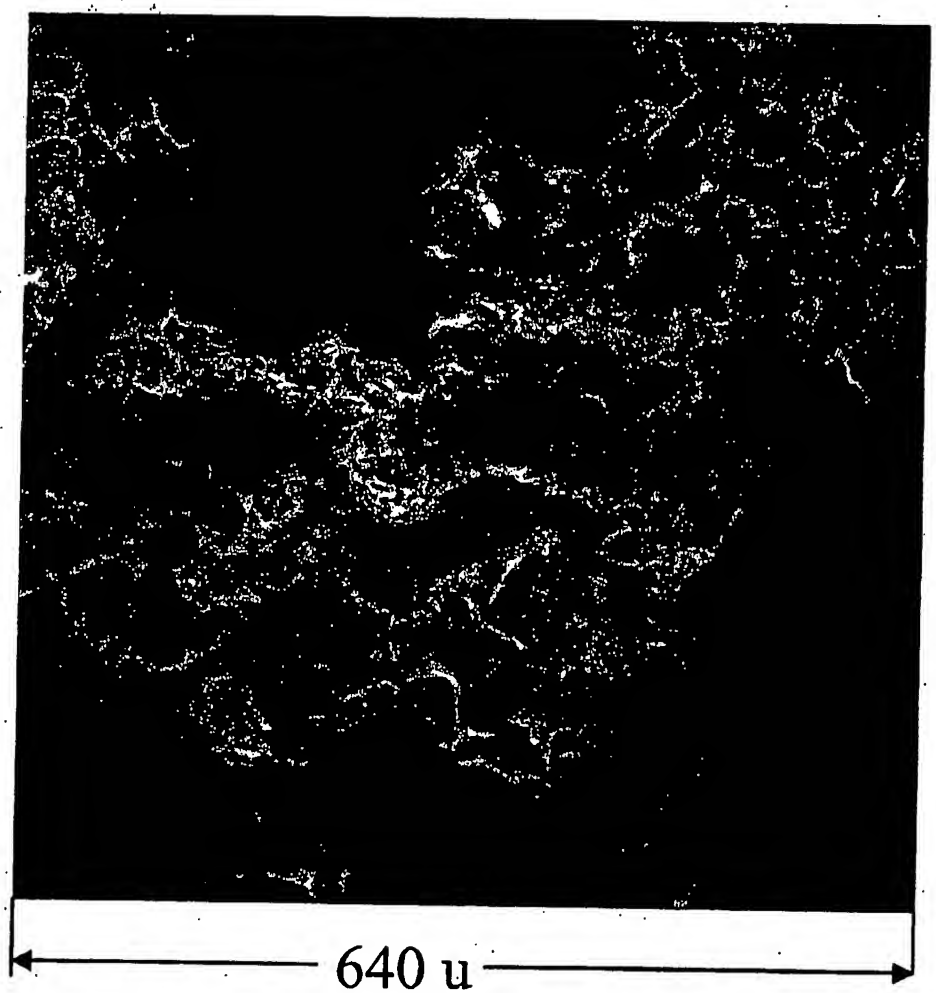


Figure 14

Sample No.	Sample Shape	Cooling Conditions	Cooling Media	Media Temp	Wetting Method
3	droplet placed on release sheet	At room temp., place droplets on to release lines. Place assembly in insulate container. Place container in -20C freezer	air	-20	direct



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Figure 23

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-70

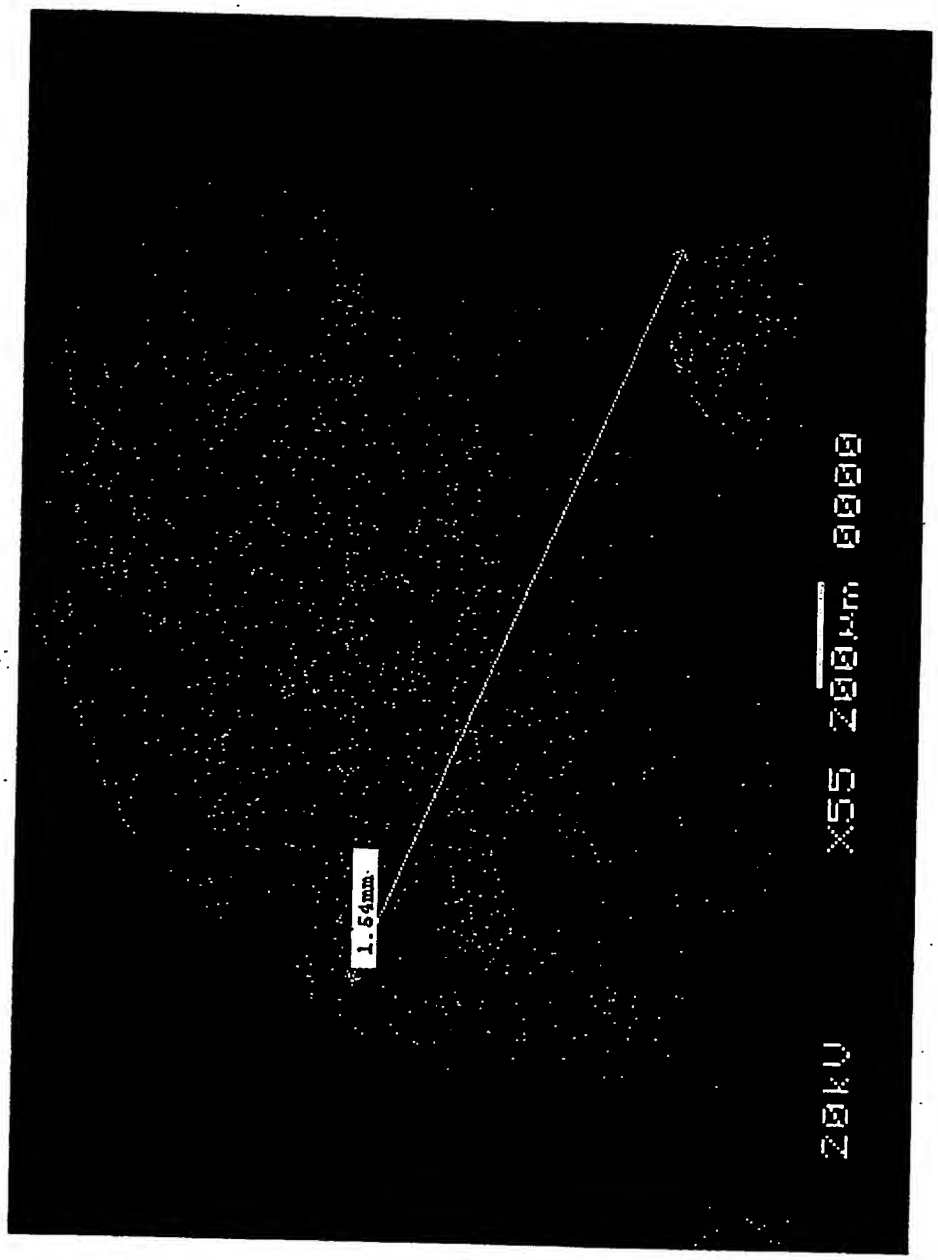


Figure 23A

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5		2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath	pentane	-70

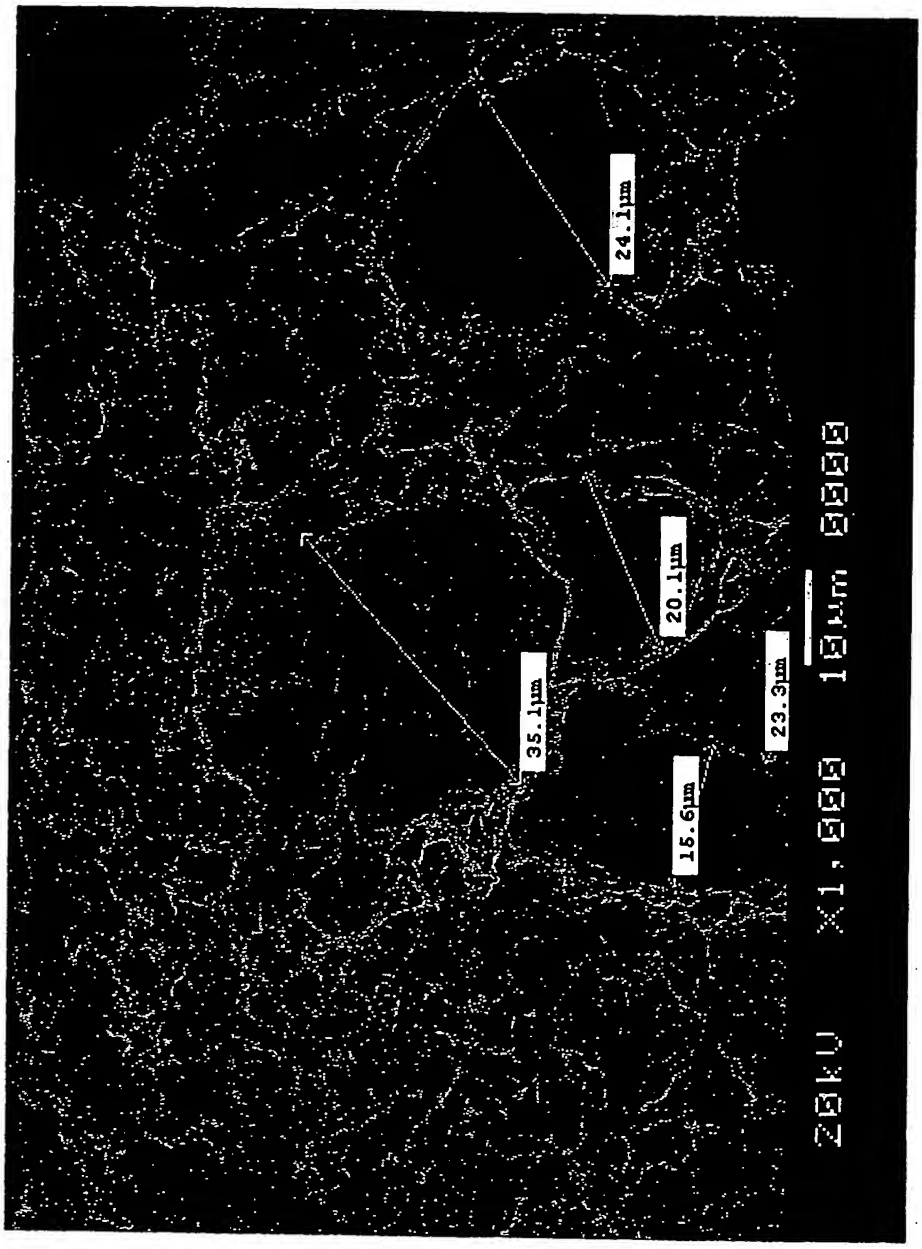
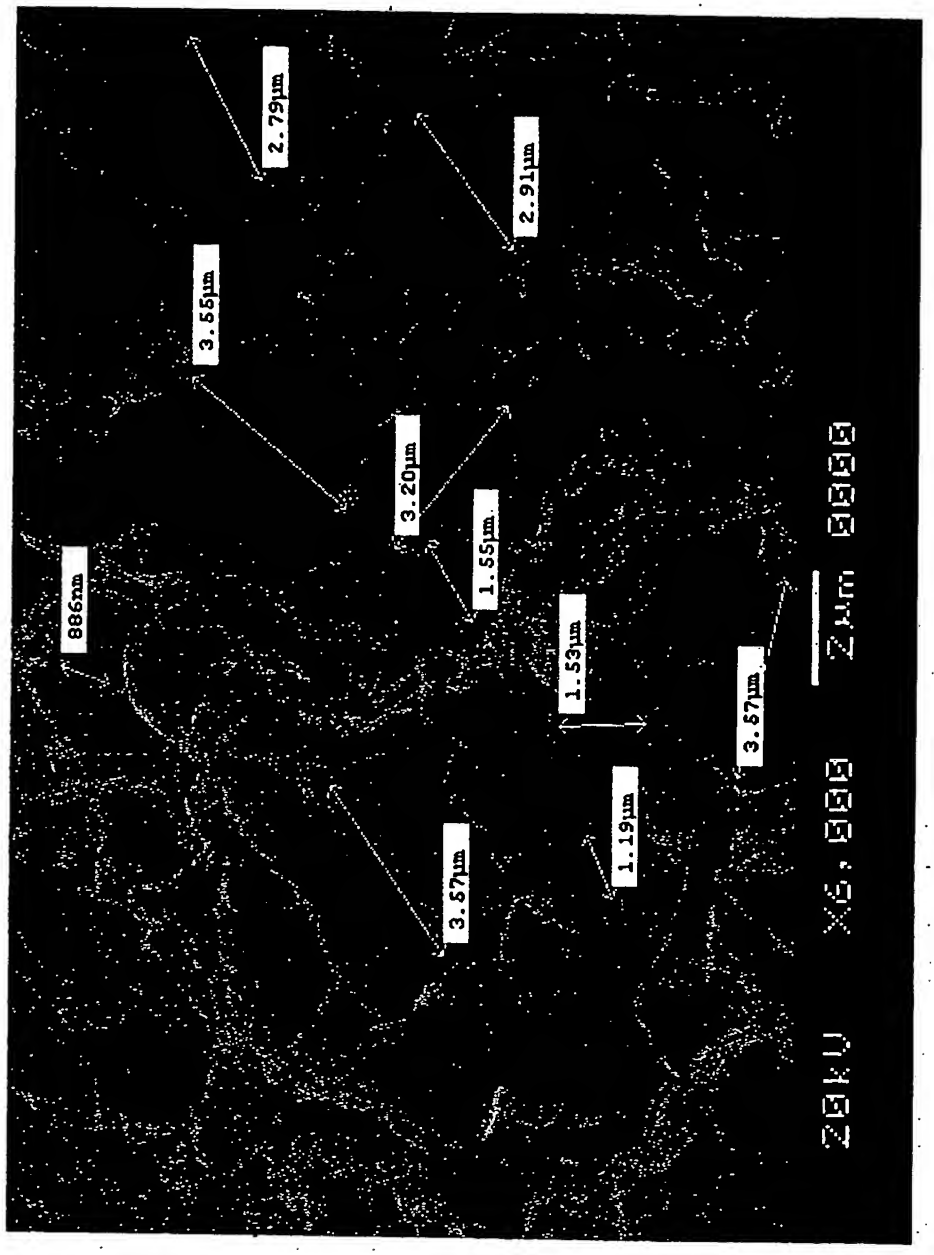


Figure 23B

Collagen Conc mg/ml	Acetic Acid Conc	Sample Shape	Cooling Conditions	Cooling Media	Media Temp C
5	2mm to .5mm droplets	Meter droplets of dispersion through no. 22 needle into coolant bath		pentane	-70



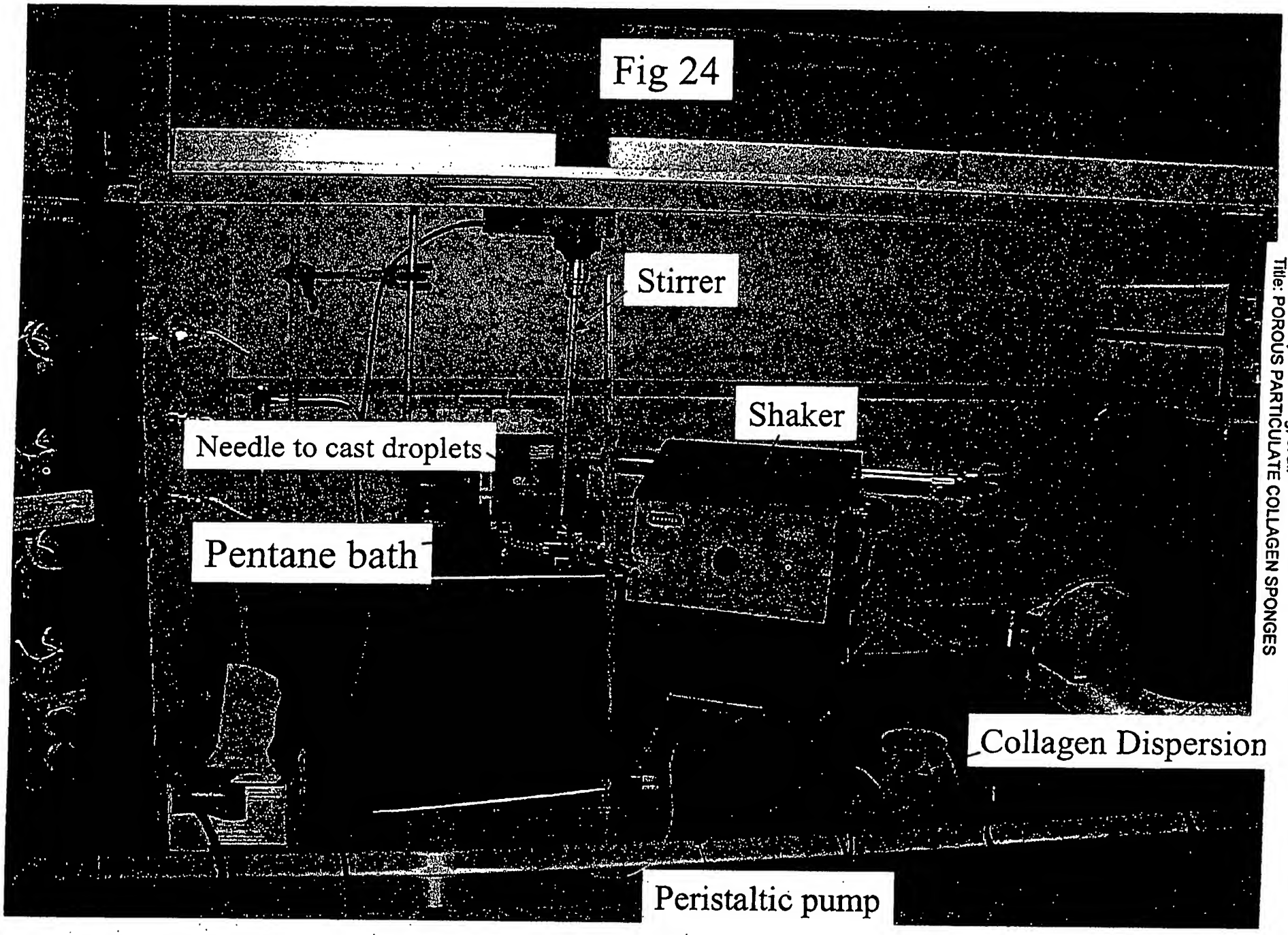


Fig 24
Stirrer
Shaker
Needle to cast droplets
Pentane bath
Collagen Dispersion
Peristaltic pump

Figure 25
Liquid N2 No. 1

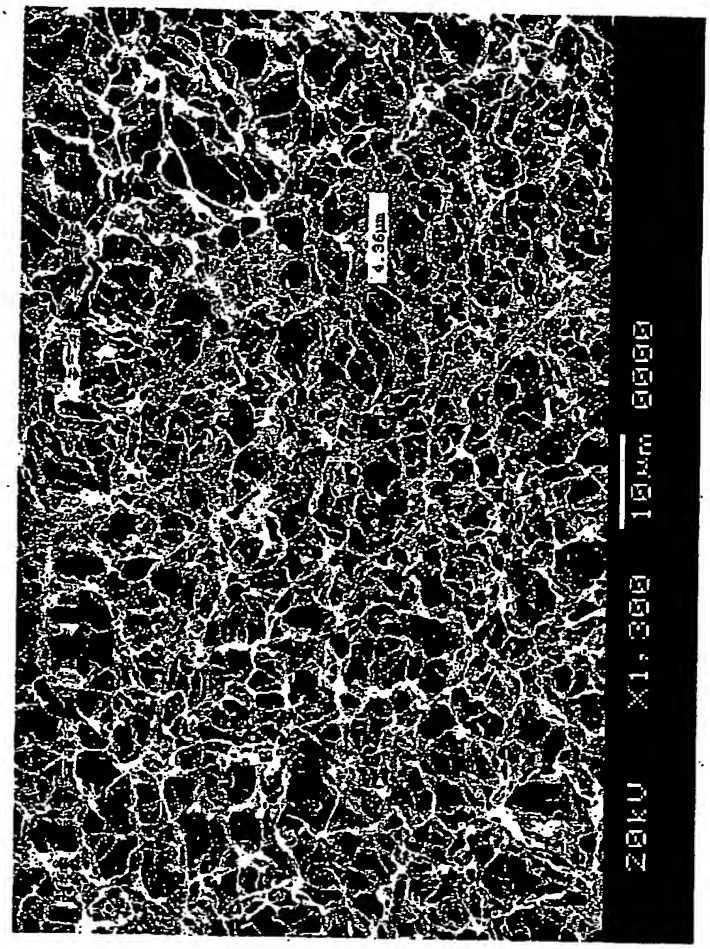


Figure 26
Liquid N2 No. 2

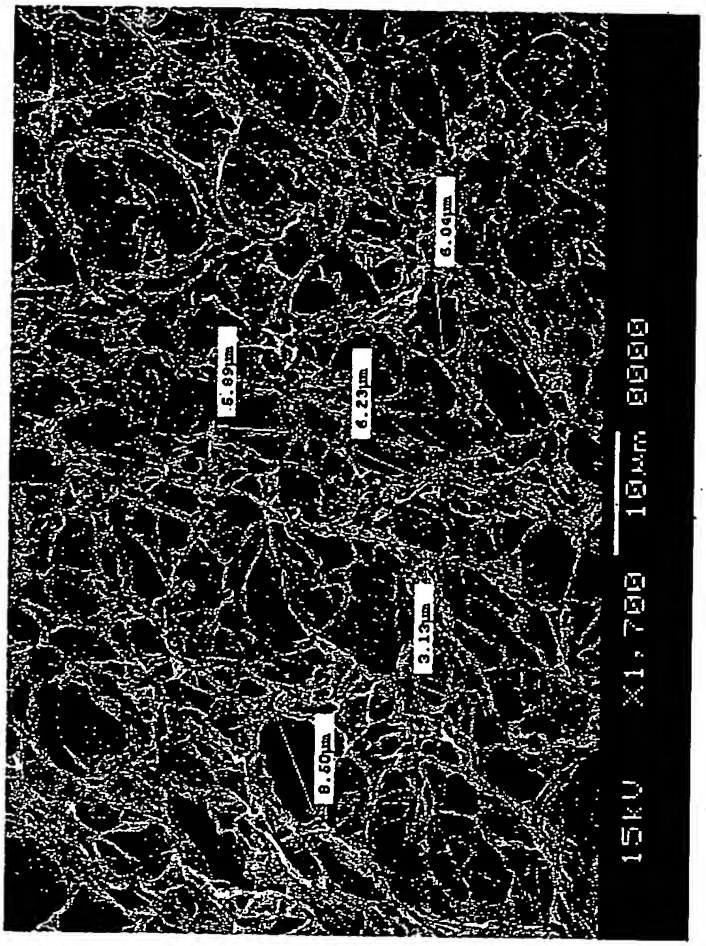


Figure 27
Liquid Pentane at –15C - No. 1

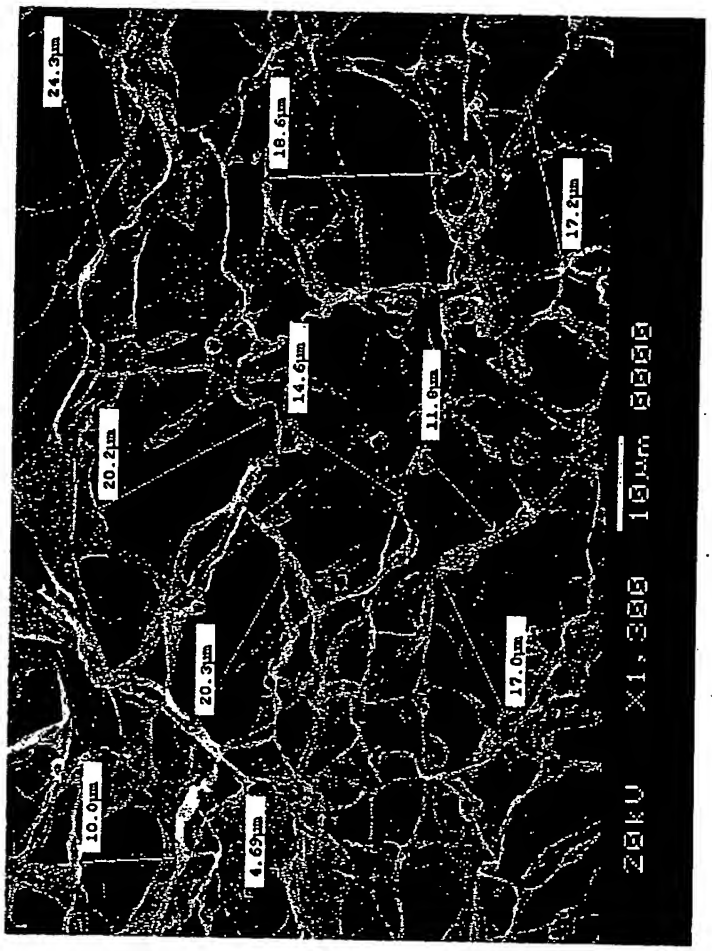


Figure 28
Liquid Pentane at –15C - No. 2

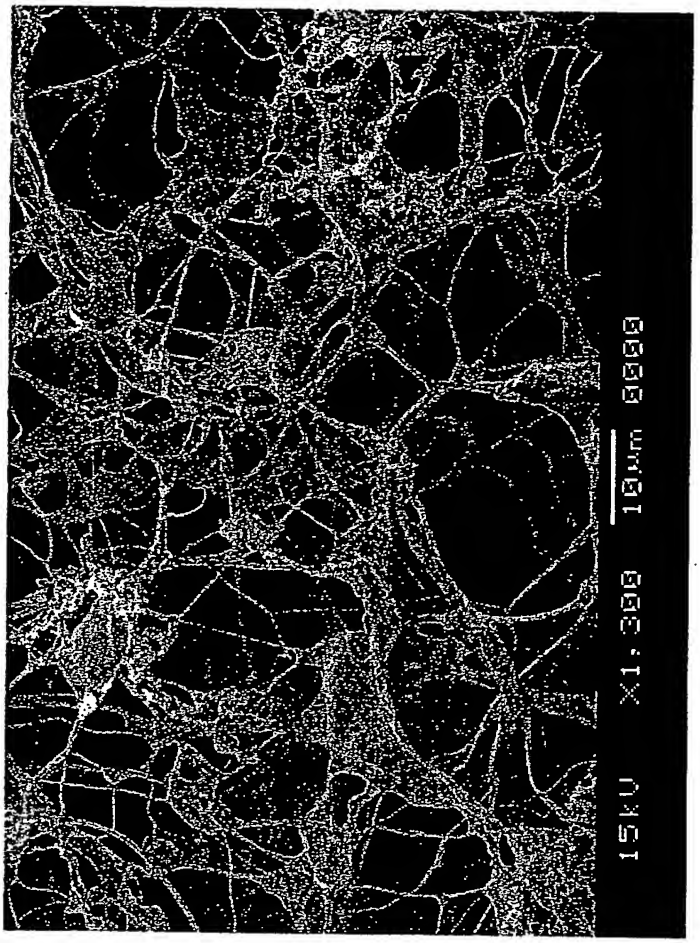


Figure 29
Liquid Pentane at –15C - No. 1

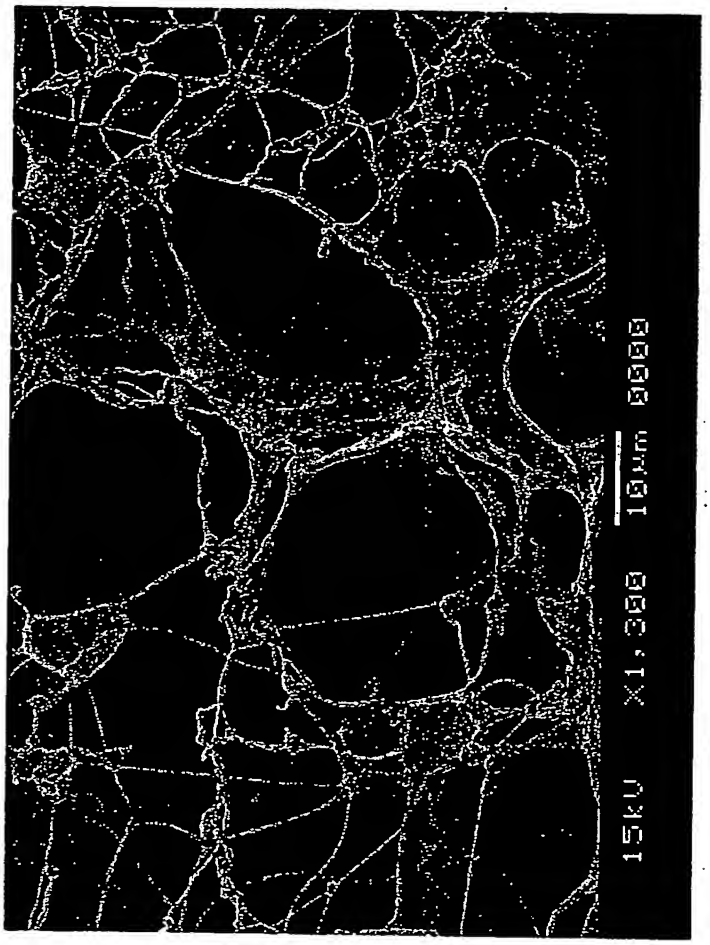
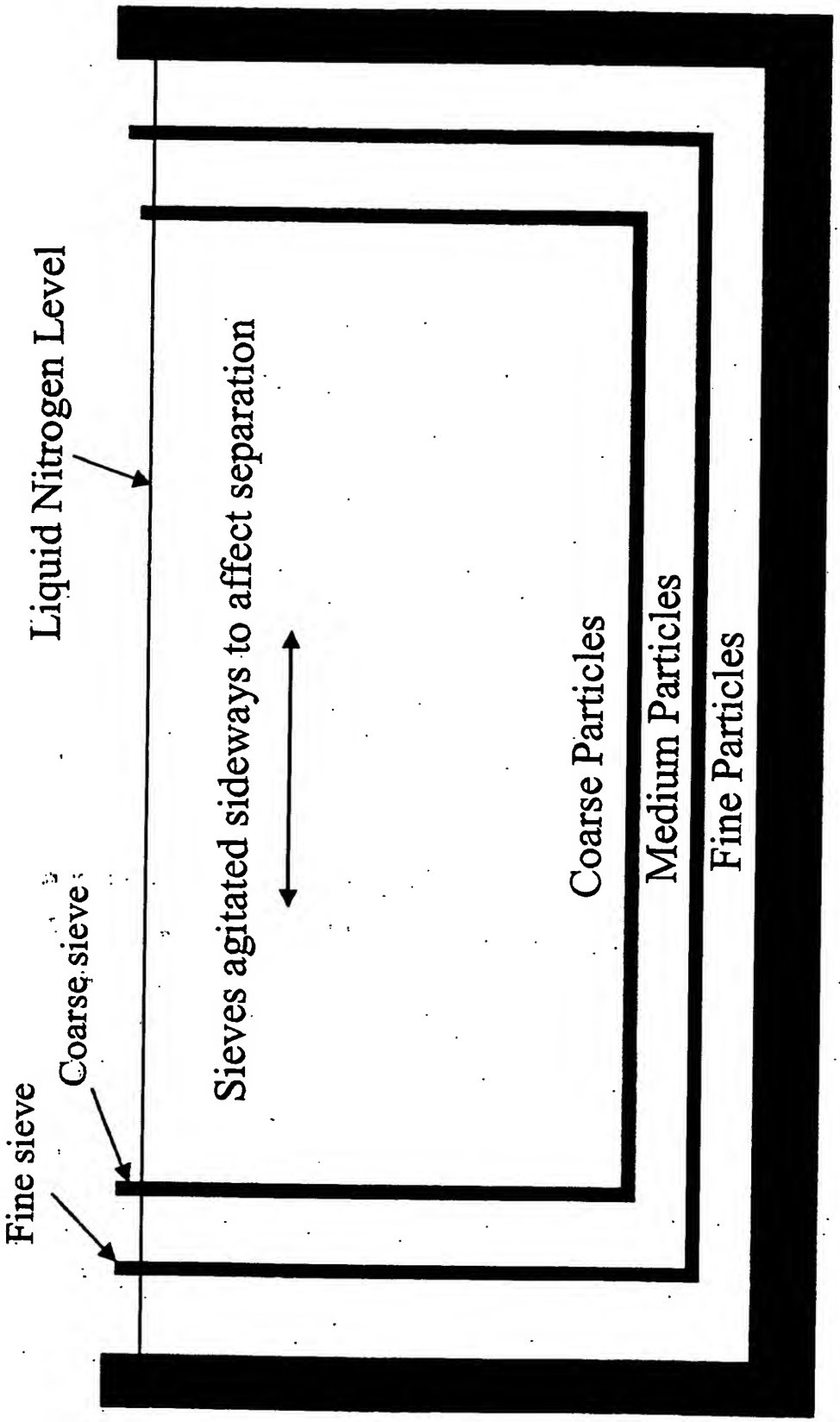
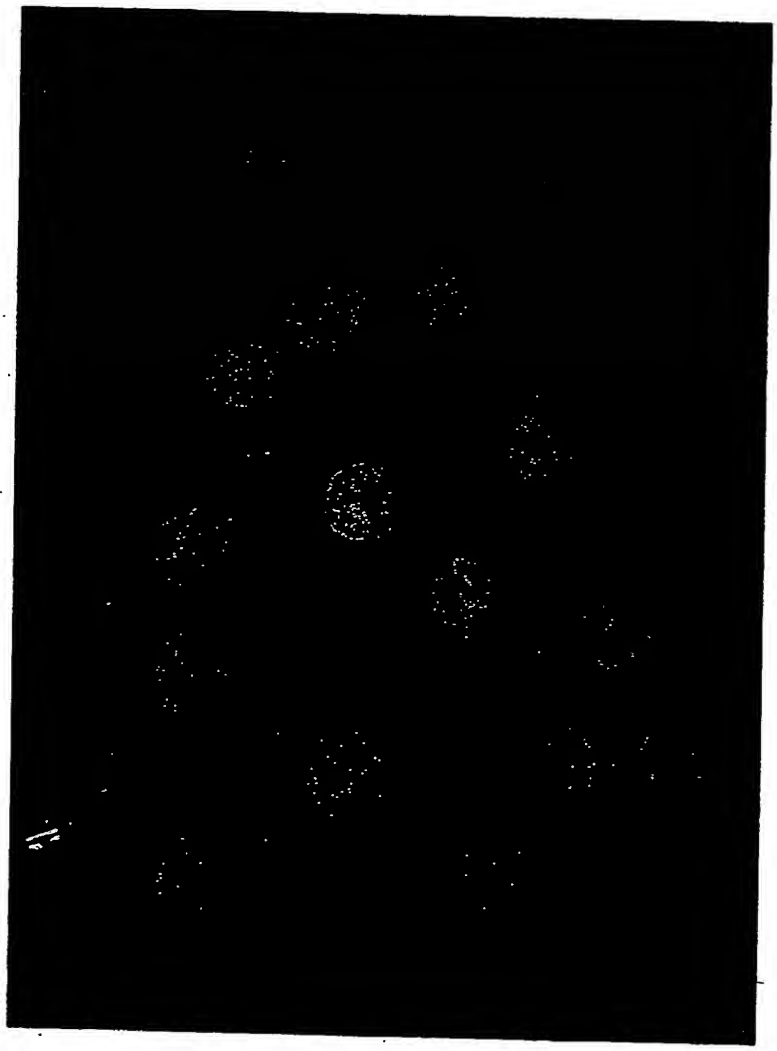


Figure 33 – Separation Device



No	Roundness
1	1.06
2	1.14
3	1.03
4	1.02
5	1.20
6	1.02
7	1.13
8	1.00
9	1.05
10	1.03
11	1.00
12	1.00
13	1.01
14	1.06
15	1.02
16	1.00

Fig 34



Roundness
2.30
1.91
1.58
1.55
1.42
1.34
1.32
1.32
1.31
1.29
1.27
1.27
1.22
1.21
1.21
1.21
1.20
1.20
1.18
1.15
1.15
1.14
1.14
1.10
1.09
1.09
1.09
1.09
1.08
1.07
1.07
1.06
1.06
1.04
1.03
1.02
1.02
1.02
1.01
1.00
1.00
1.00
1.00

Fig 35

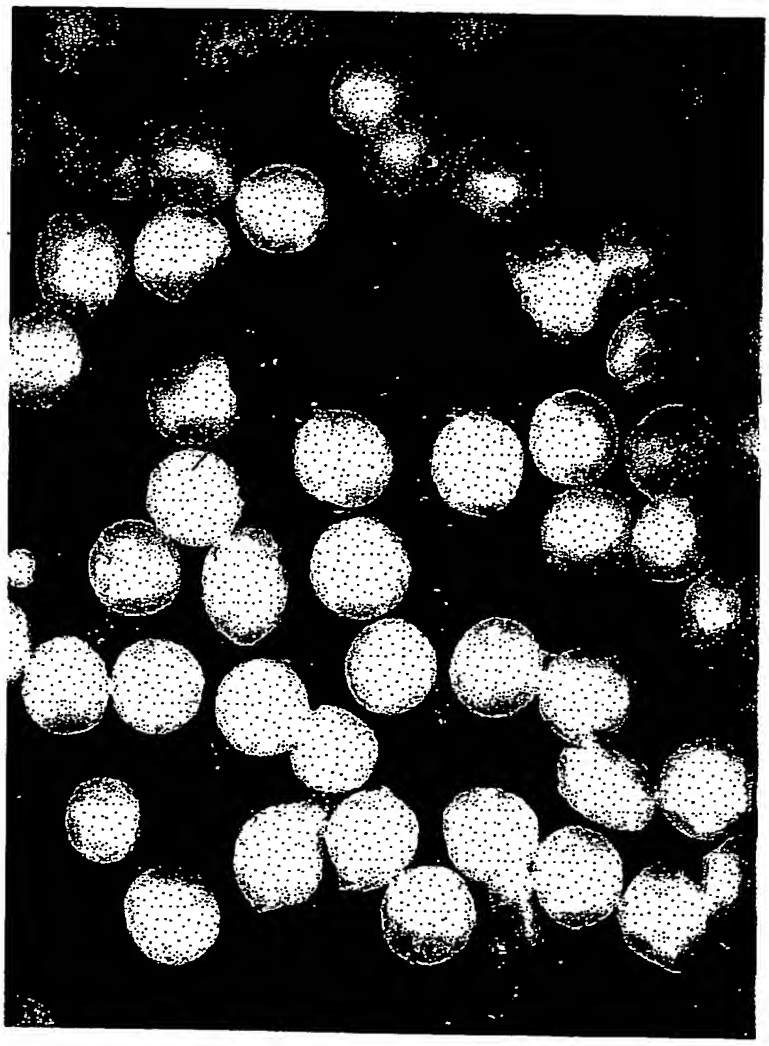
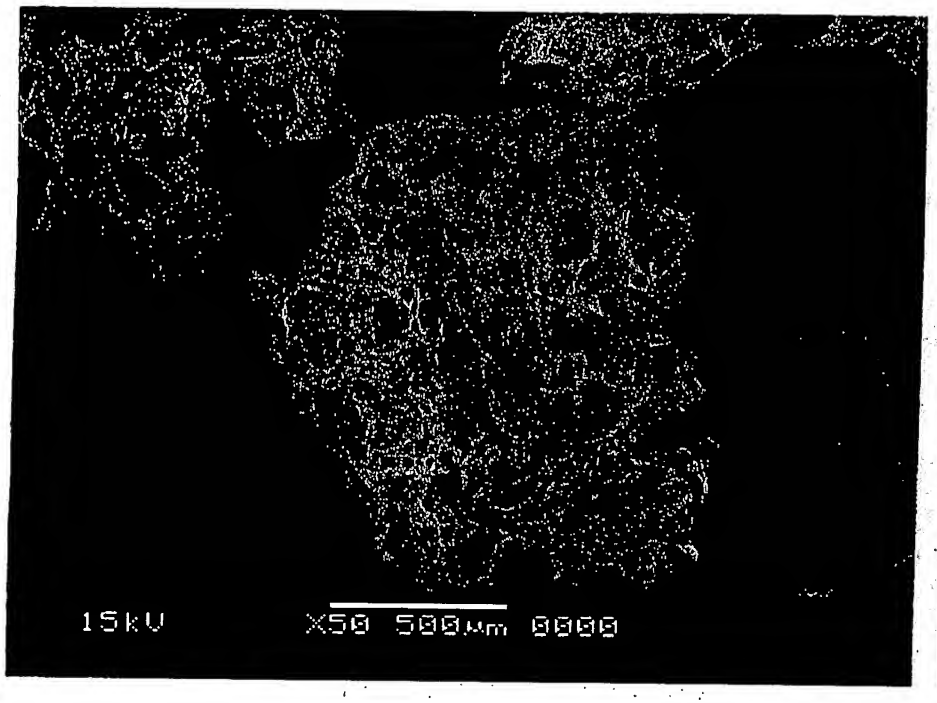


Fig 37A – Non Spherical Particulate Spheres SEM



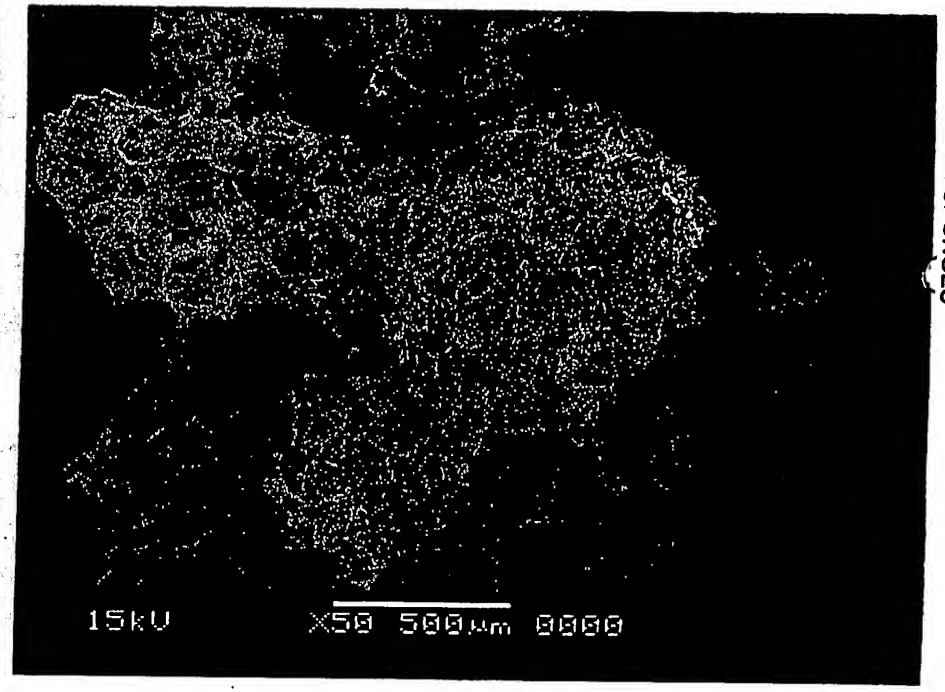
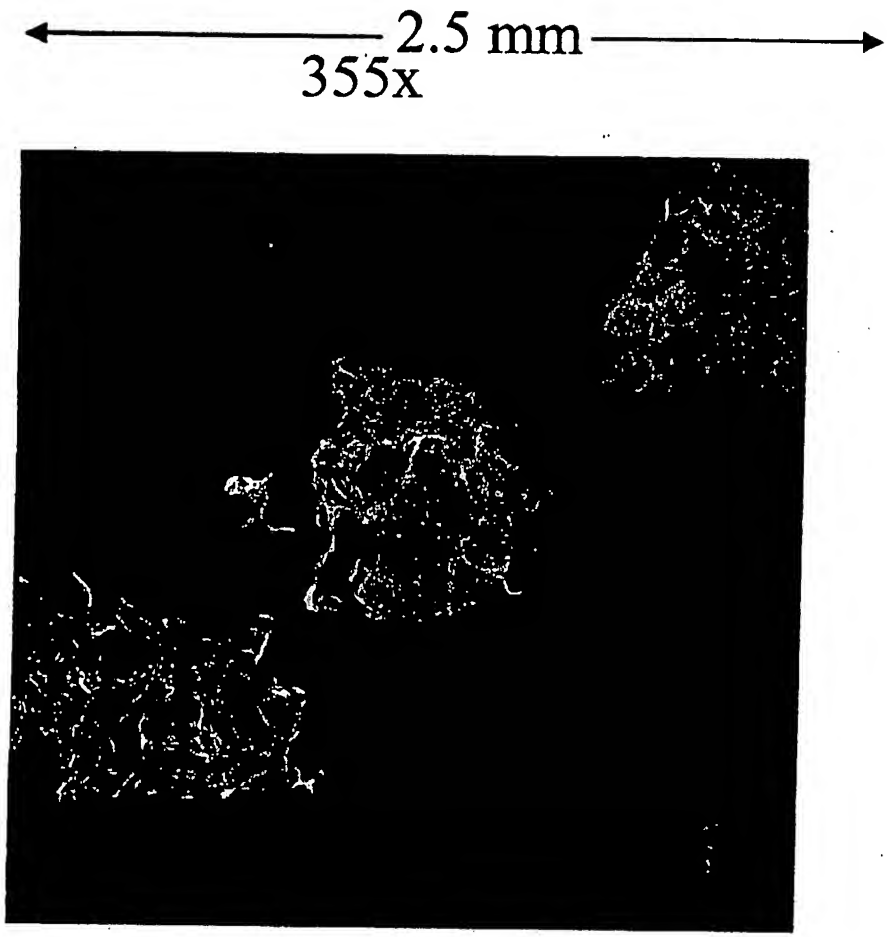
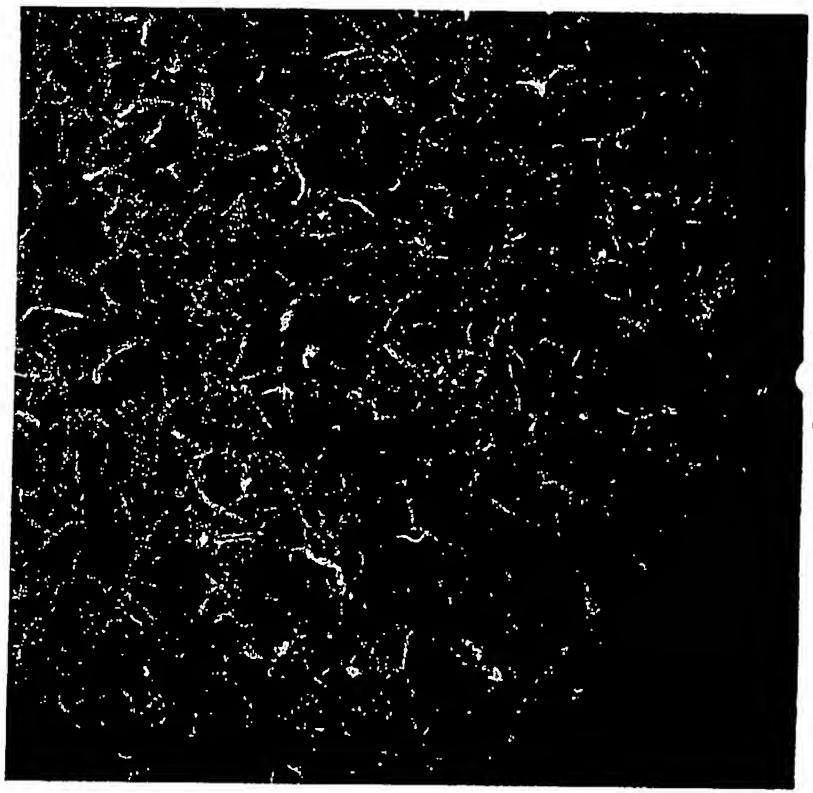
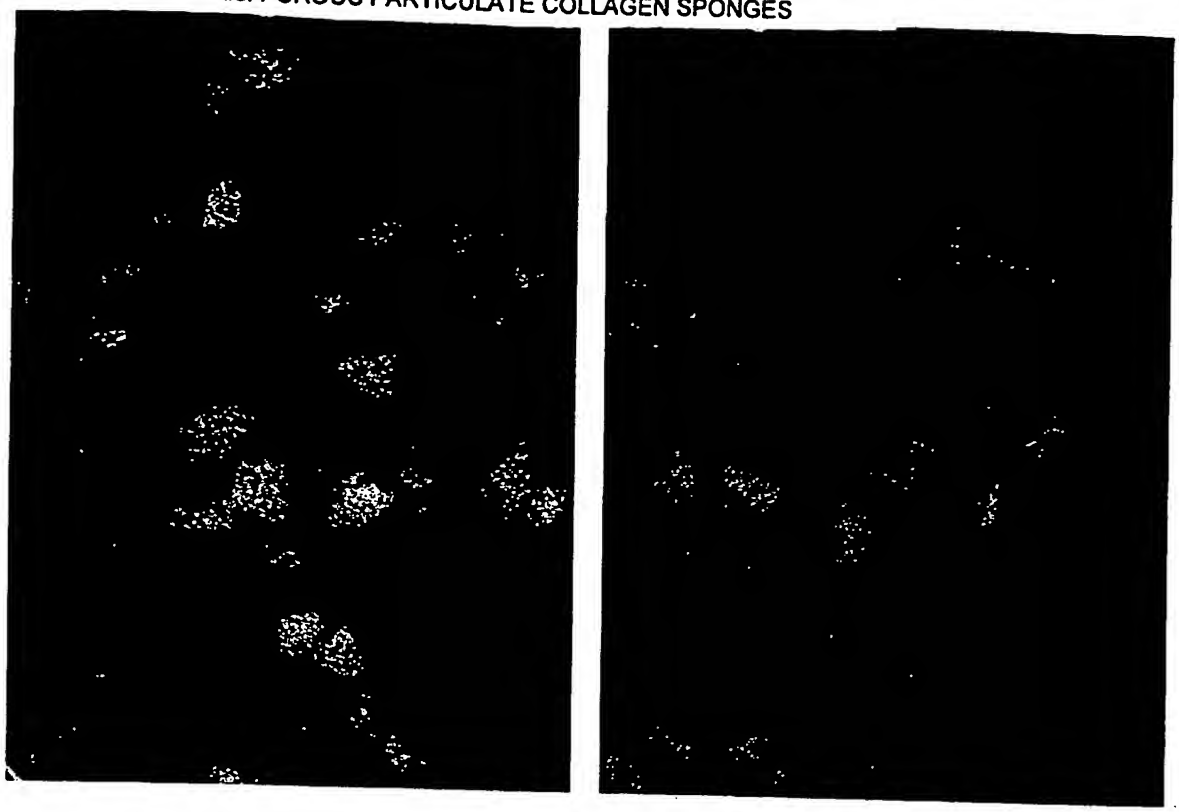


Fig 37B – Wetted Non Spherical Particulate Spheres Confocal





App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2



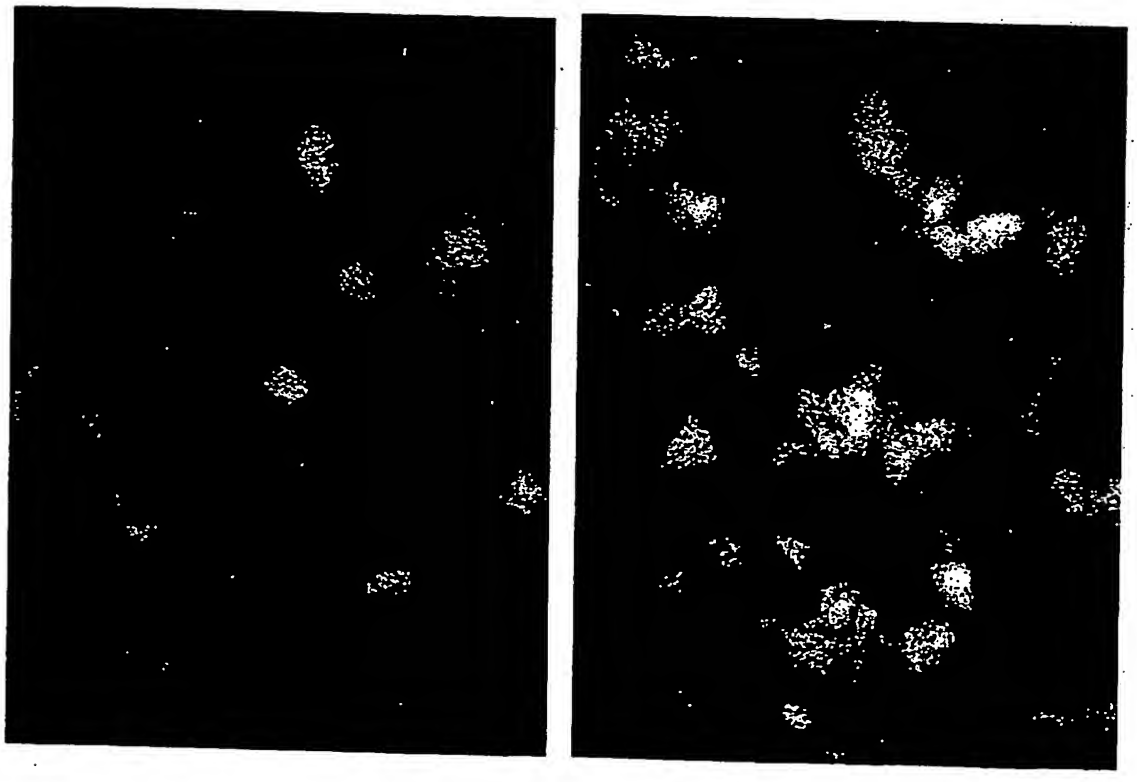
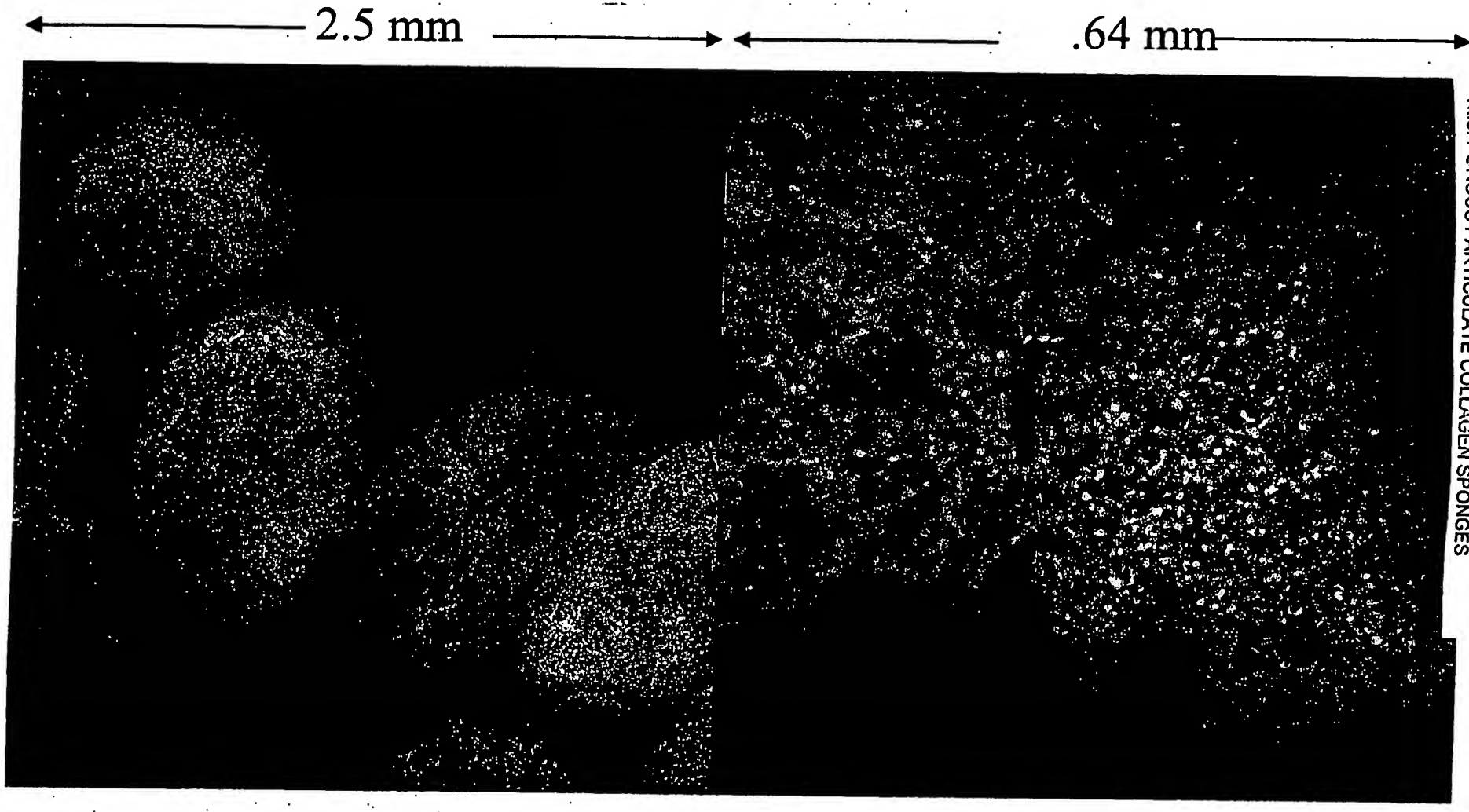


Fig 38

Fig. 39
Porcine Fibroblasts Cultured in vitro
On Porous Collagen Spheres
Pentane Spheres



App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

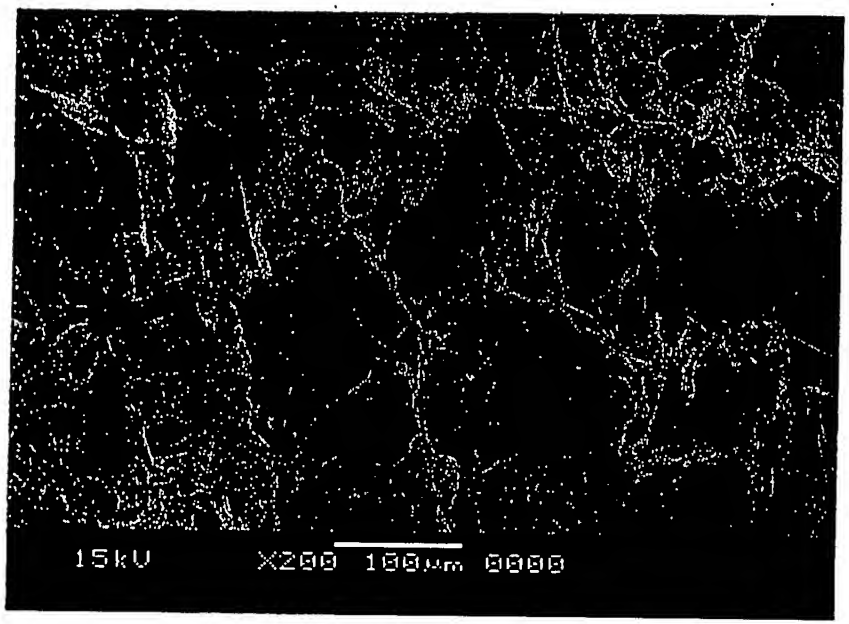
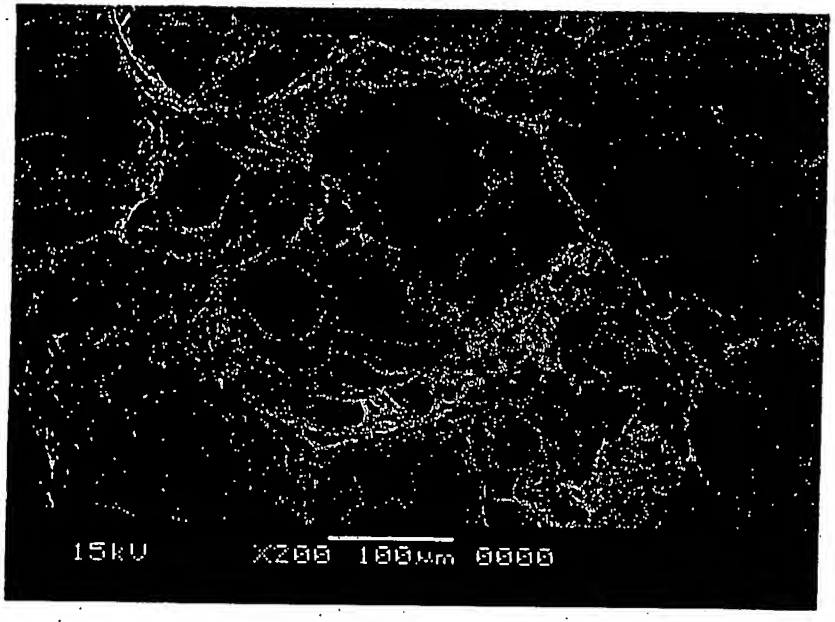
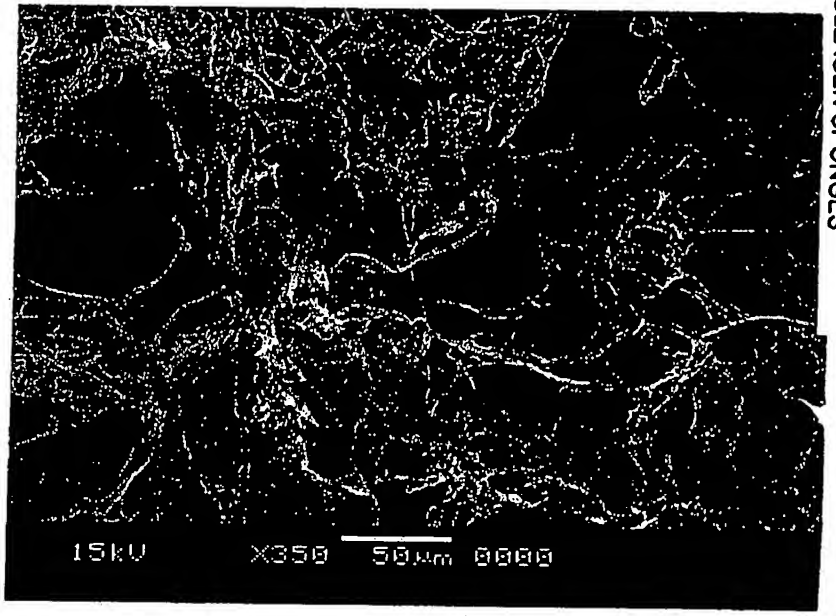


Fig 41
Images forPore Size Distribution
Frozen in Air @ -20C
Ground
Sieved through 1.5mm Screen
And retained on .5mm screen





App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

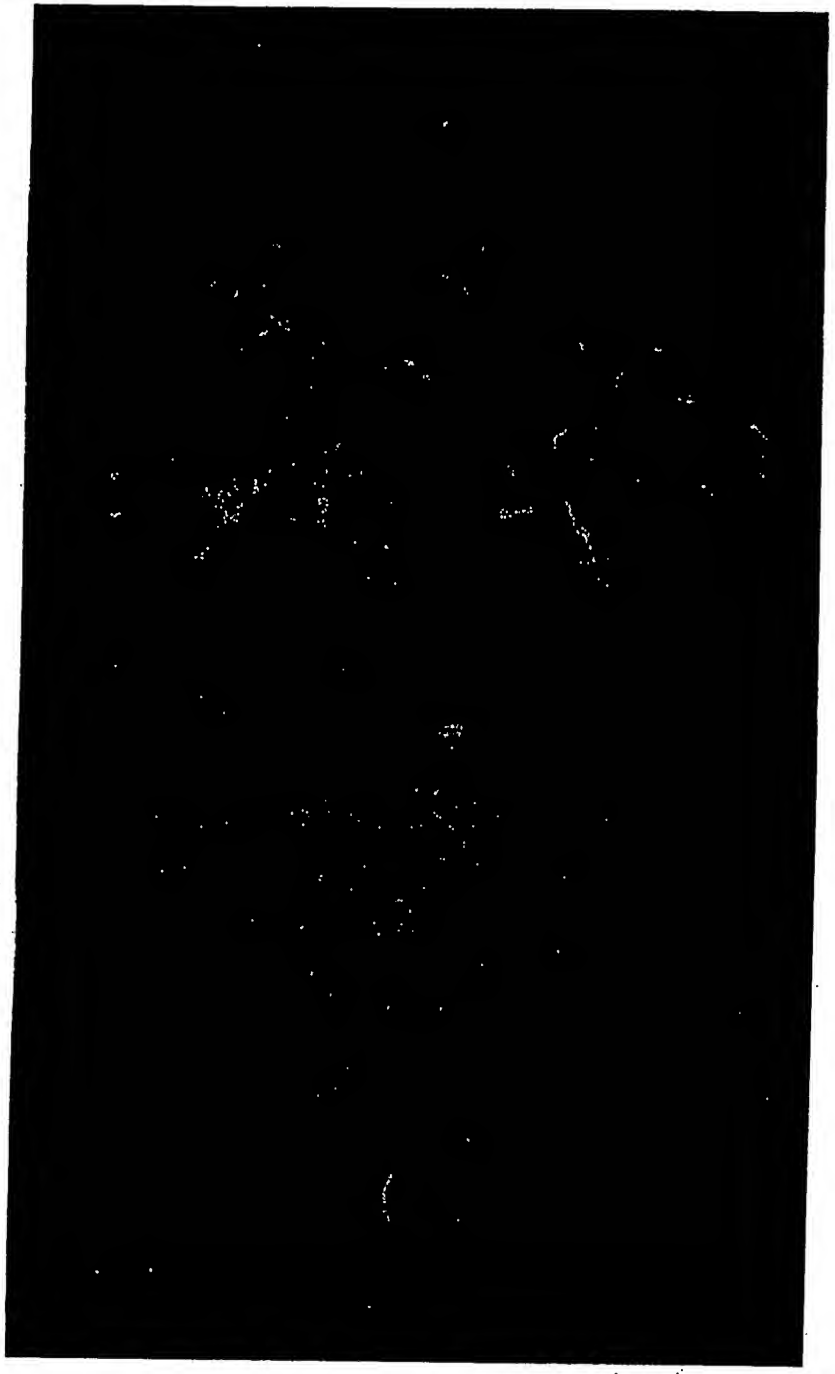


Fig 42
Fibroblasts/Spheres/No Gel
Liquid N2 Spheres

5c

5d

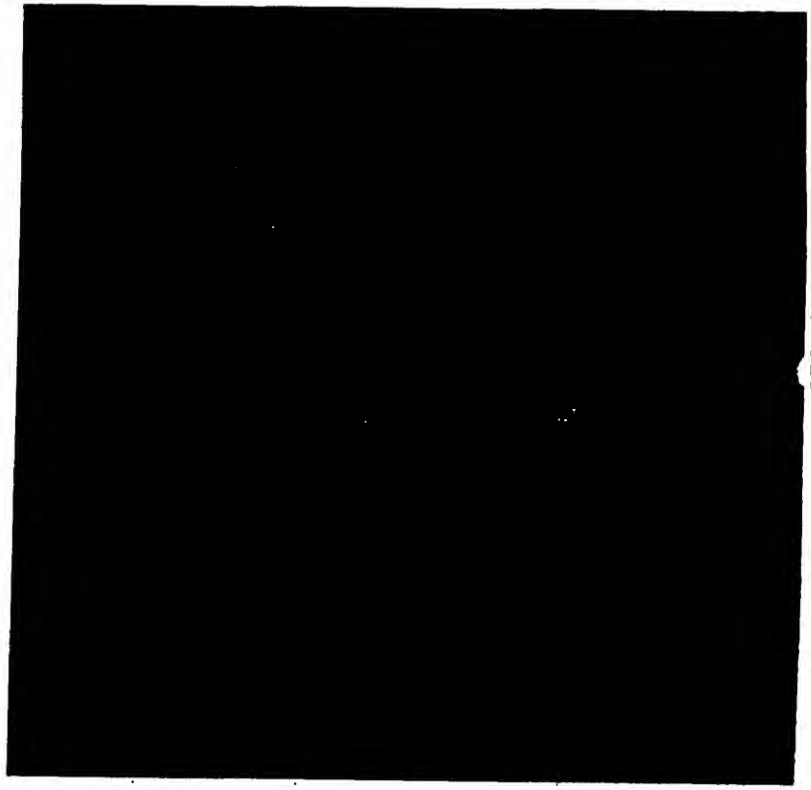


Fig 50

App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

Fig 51
Fibroblasts/Particles/No Gel
Cooled in air @ -20C

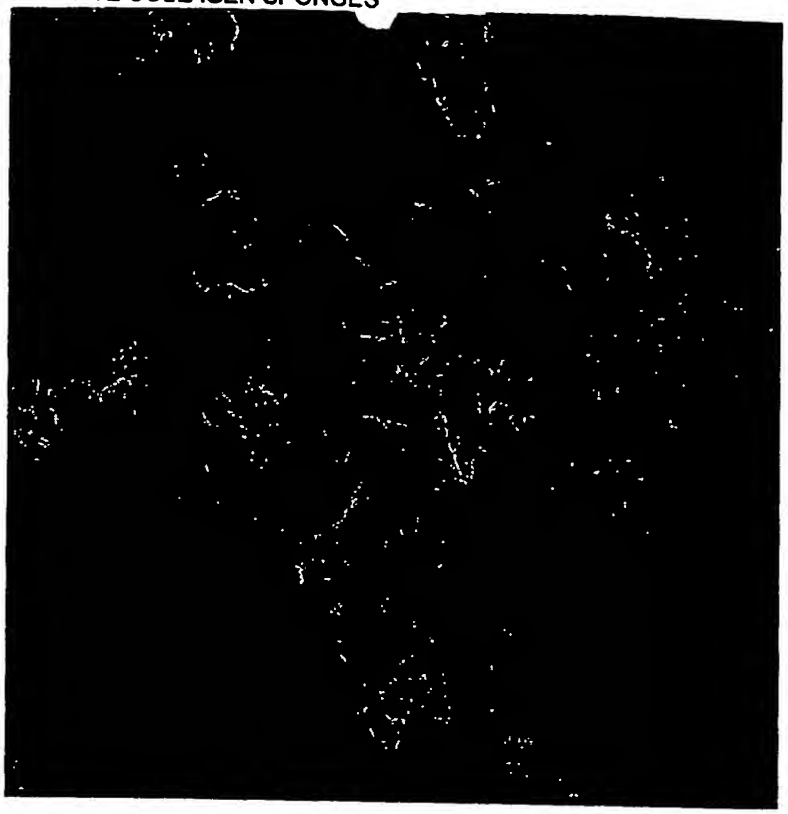


Fig 52
Collagen/C6S (4/1)

App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GRN-005-2

44-1 Premixed

45 Mixed in Tray

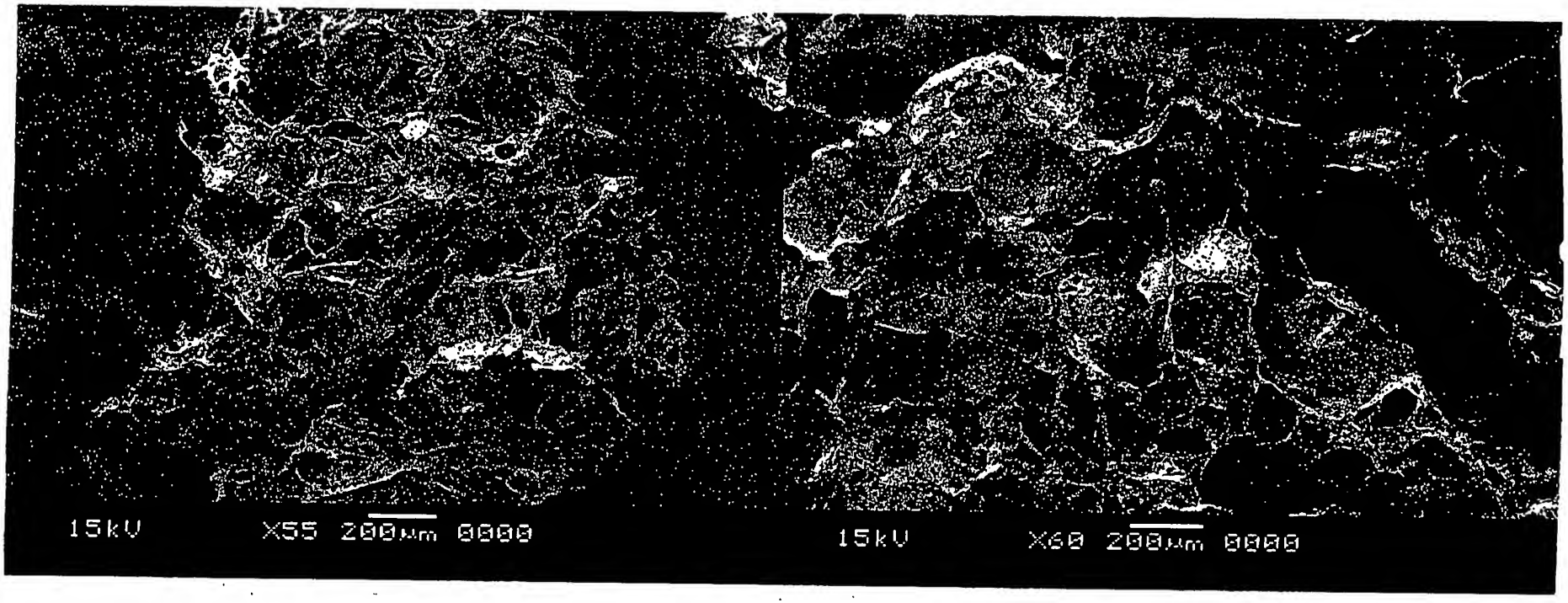
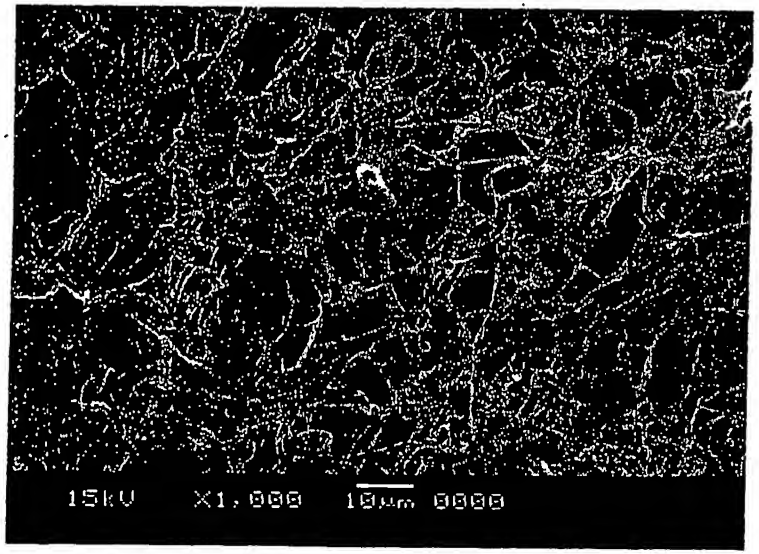
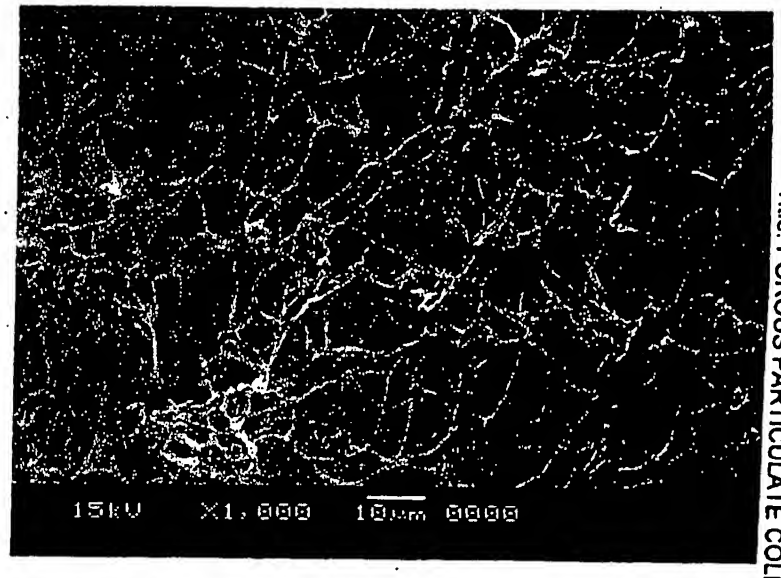
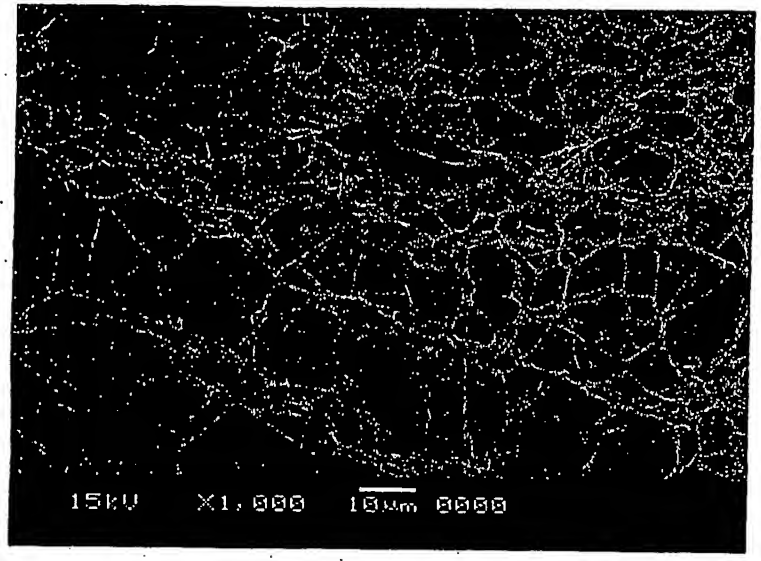


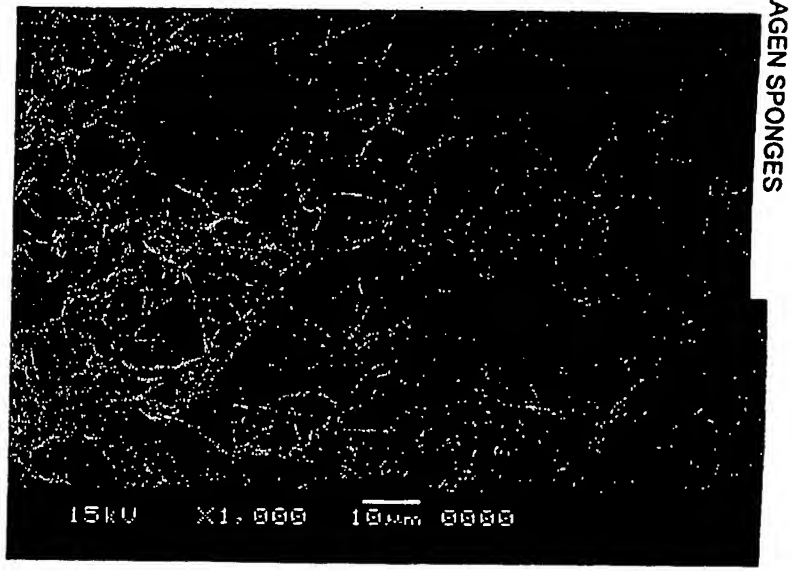
Fig. 53

Particles Frozen in Liquid Nitrogen and Ground in Liquin Nitrogen







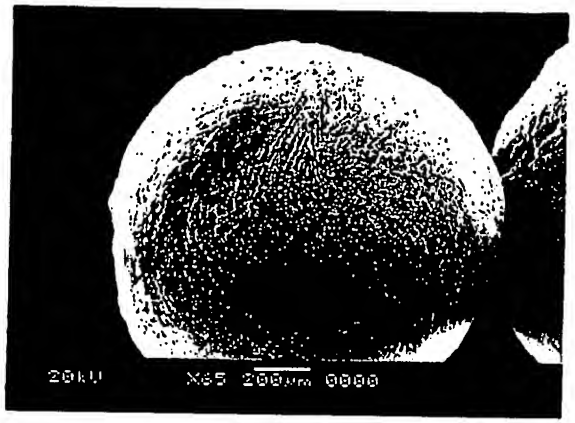


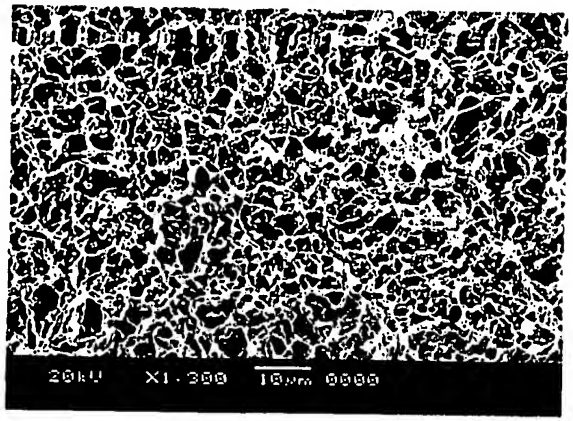
App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

Docket No.: GRN-005-2

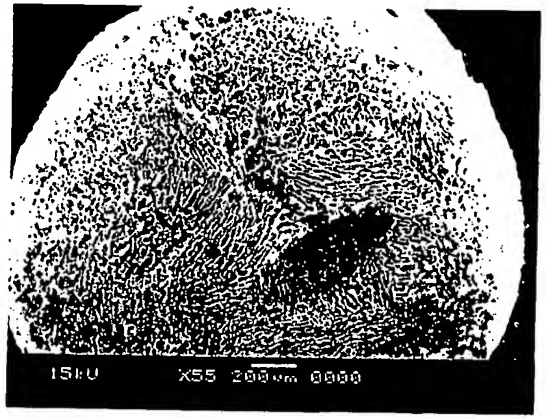
Figure 54
Dry Particulate Collagen Sponges

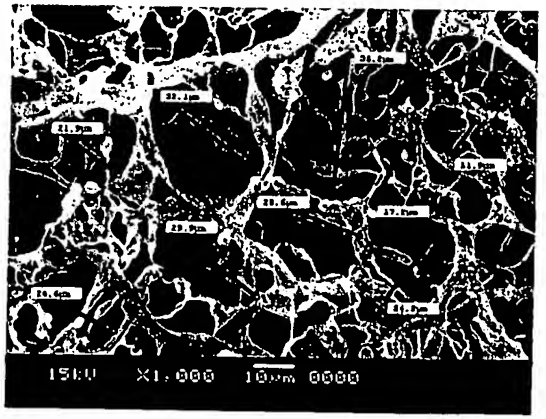
Particles Cast in Liq. N2 – No.1



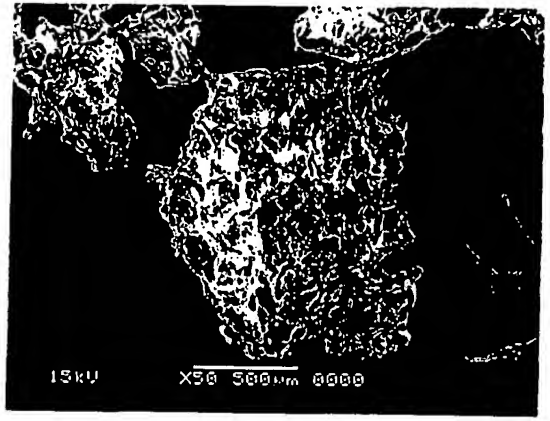


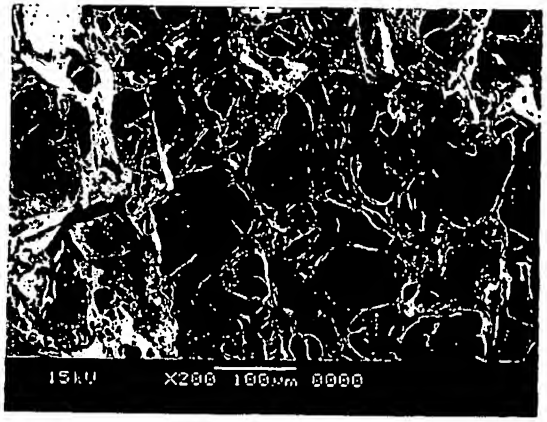
Particles Cast in Pentane @ -15C – No. 2





Particles Cast in Air @ -20C – No. 3

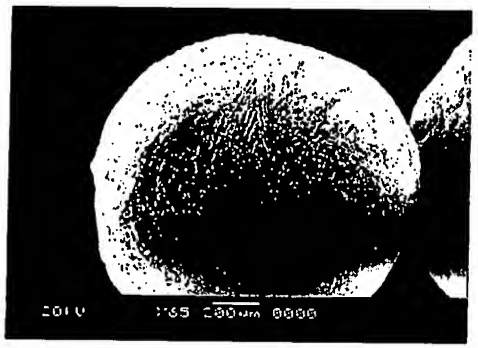




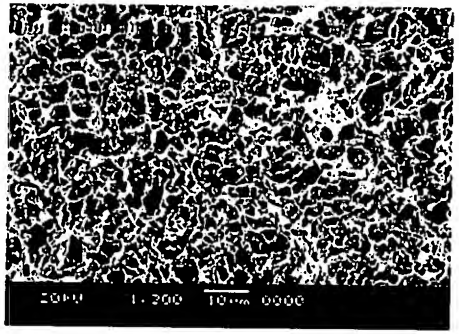
App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

Docket No.: GRN-005-2

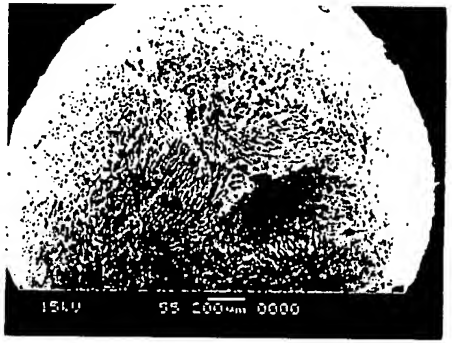
Figure 54 (cont-1) – Dry Sponges - SEM



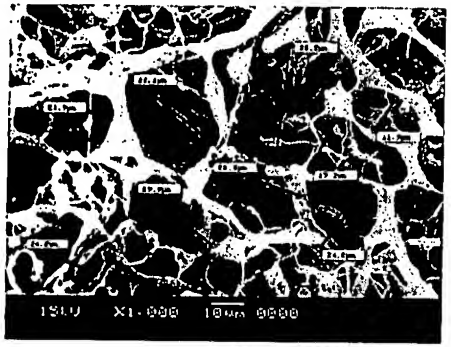
A



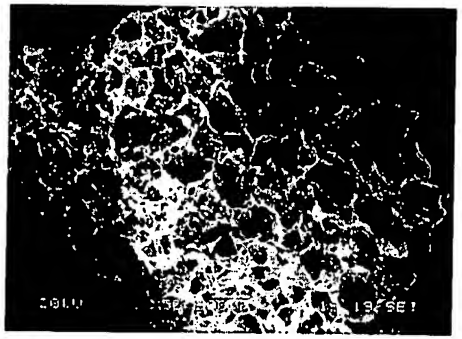
B



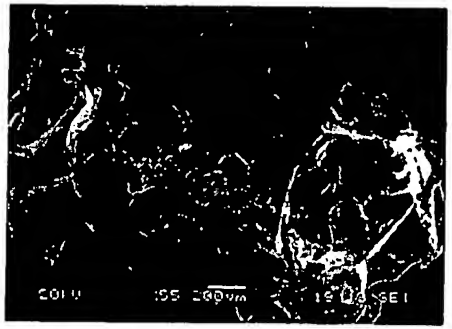
C



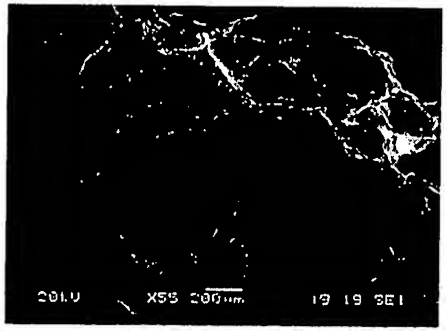
D



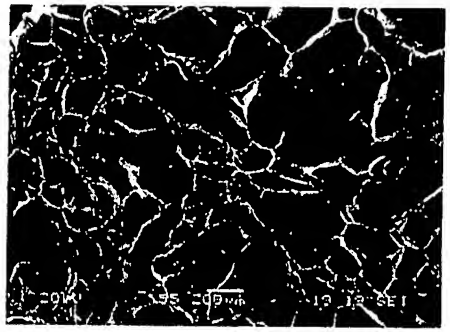
E



F



G



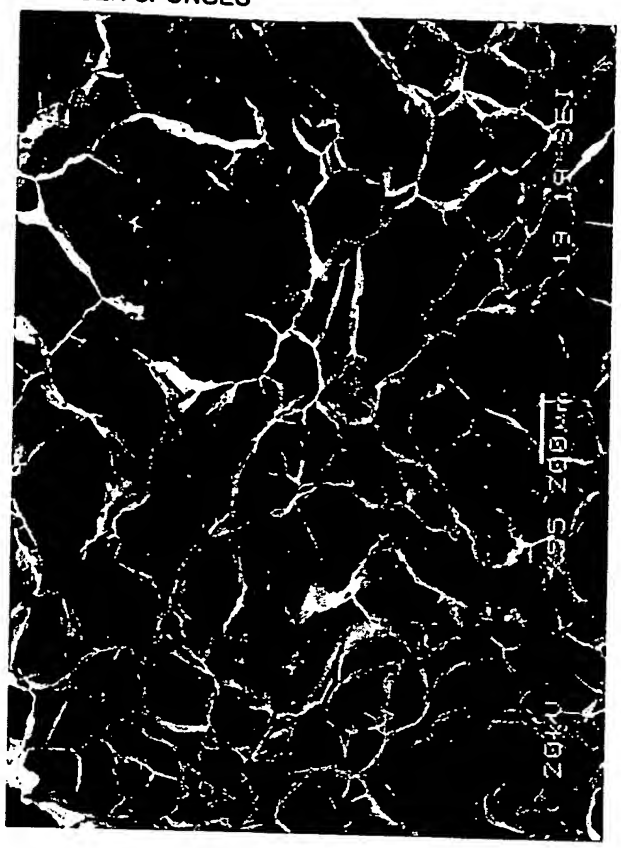
H

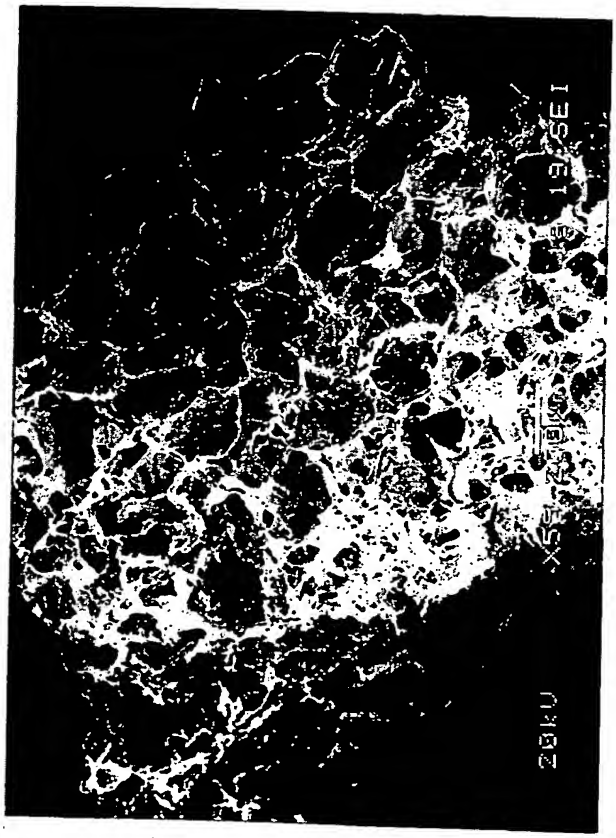
No. 1

App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

Docket No.: GRN-005-2

Figure 54 (cont-2)





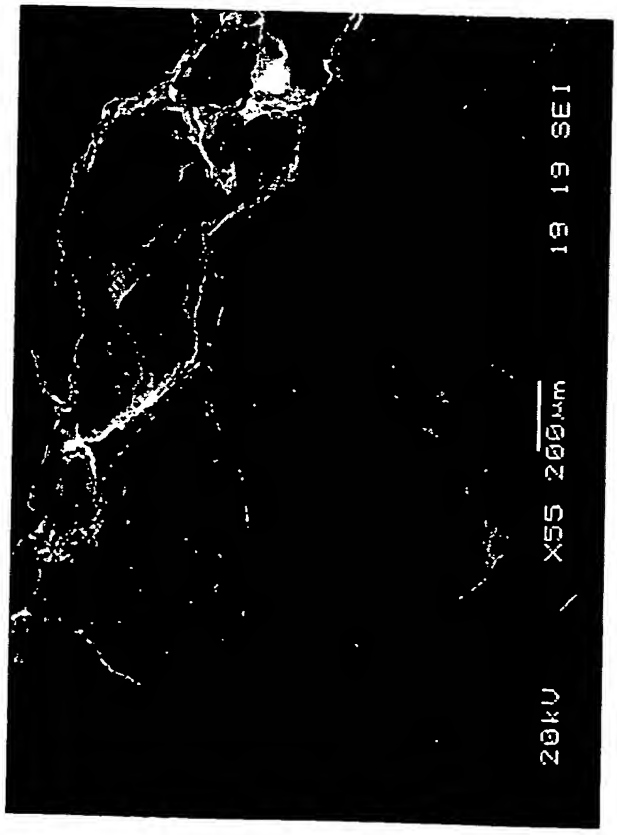


Figure 55
Effects of Wetting Procedure on Sample No. 3

2 Step Wetting Process

Dry Samples

wet

Direct Wetting in PBS

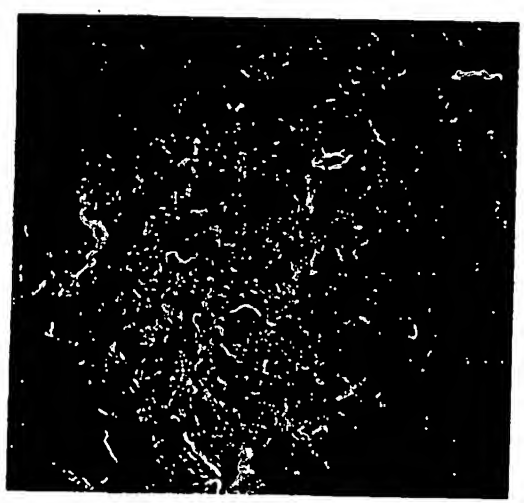
Dry Samples

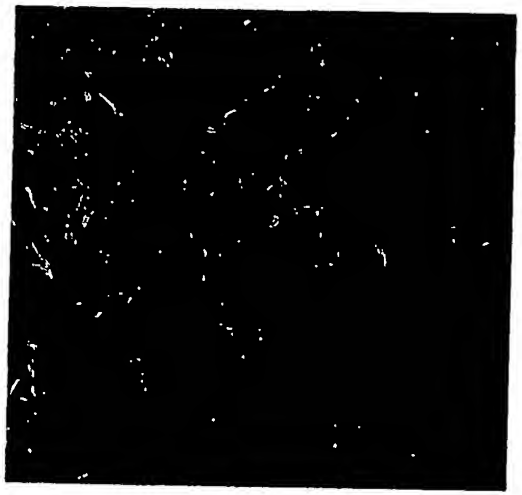
wet

App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES
Docket No.: GKN-005-2

Fig 56 - Confocal Microscopy Images of Wetted Particles

9 Step Wetting Procedure

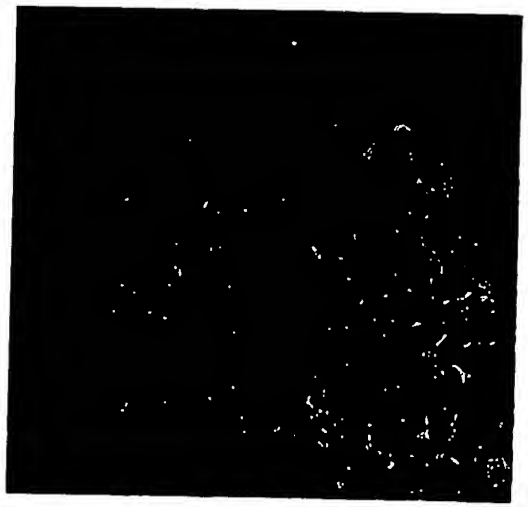




Wetting Directly in PBS

Particles Cast in Liq. N2 – No.1

Particles Cast in Pentane @ -15C – No. 2



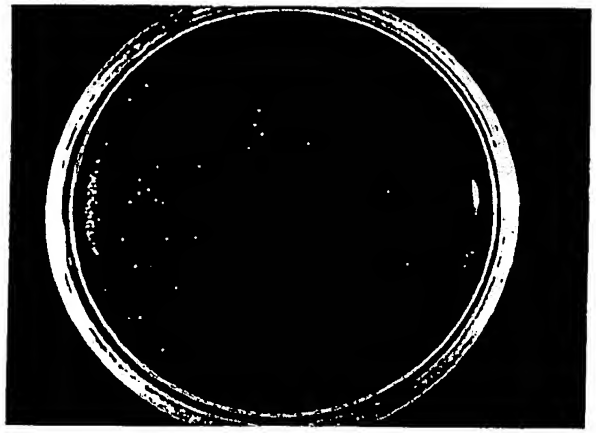
Particles Cast in Air @ -20C – No. 3

App No.: Not Yet Assigned
Inventor: Roy H.L. Pang, et al.
Title: POROUS PARTICULATE COLLAGEN SPONGES

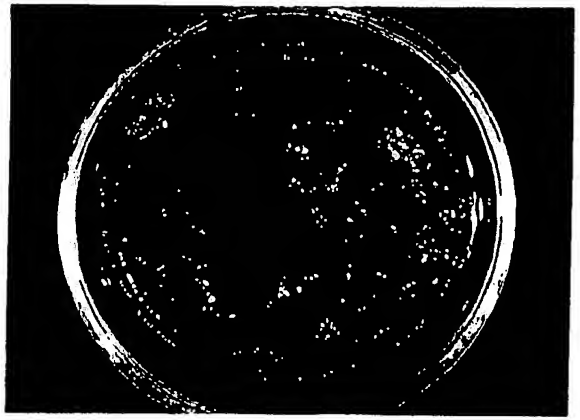
Docket No.: GRN-005-2

Fig. 57
Effects of Wetting Procedure on Sponge Sheets

2 Step Process



Direct Wetting in 70% ethanol



Direct Wetting in PBS

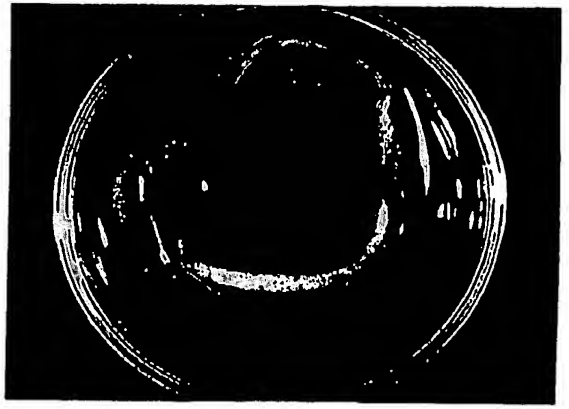
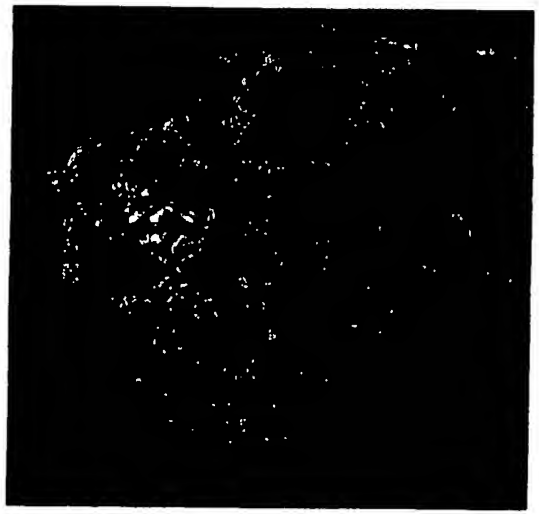


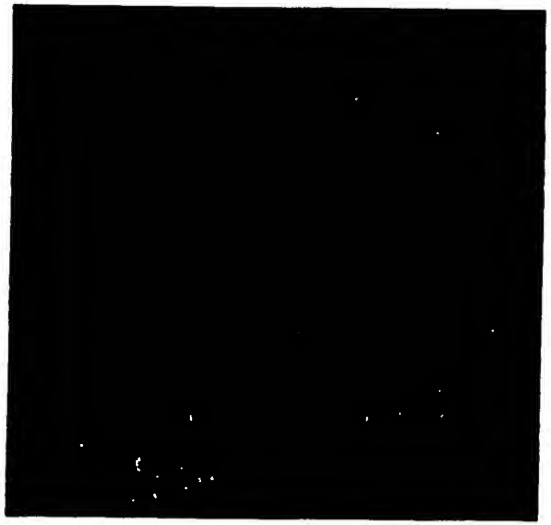
Fig 58
Proliferation of Porcine Fibroblasts on Collagen Particles

Day 6 (Dish)



Particles Cast in Liq. N2 – No.1

Day 10 (Spinner Flask)



Particles Cast in Pentane @ -15C – No. 2

Day 10 (Spinner Flask)

Particles Cast in Air @ -20C – No. 3

Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	POROUS PARTICULATE COLLAGEN SPONGES
Attorney Docket Number::	GRN-005-2
Request for Early Publication?::	No
Request for Non-Publication?::	No
Small Entity?::	No
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Roy
Middle Name::	H.L.
Family Name::	Pang
City of Residence::	Etna
State or Province of Residence::	NH
Country of Residence::	US
Street of mailing address::	15 Partridge Road
City of mailing address::	Etna
State or Province of mailing address::	NH
Postal or Zip Code of mailing address::	03750

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Robert
Middle Name::	A.
Family Name::	Wiercinski
City of Residence::	Lincoln
State or Province of Residence::	MA
Country of Residence::	US
Street of mailing address::	29 Brooks Road
City of mailing address::	Lincoln
State or Province of mailing address::	MA
Postal or Zip Code of mailing address::	01773

Applicant Authority Type::	Inventor
Status::	Full Capacity
Given Name::	Dona
Family Name::	Hevroni
City of Residence::	Lexington
State or Province of Residence::	MA
Country of Residence::	US
Street of mailing address::	425 Woburn Street, Apt. 45
City of mailing address::	Lexington
State or Province of mailing address::	MA
Postal or Zip Code of mailing address::	02420

Correspondence Information

Correspondence Customer Number:: 00959

Representative Information

Representative Customer Number:: 00959

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/010564

International filing date: 05 April 2004 (05.04.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/513,922
Filing date: 23 October 2003 (23.10.2003)

Date of receipt at the International Bureau: 15 September 2004 (15.09.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse